Effect of Maternal Age on Transcriptome of Granulosa Cells from Bovine Dominant Follicles

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ABSTRACT

Advanced maternal age has been shown to influence follicular and luteal dynamics in bovine ovary resulting in reduced fertility. The overall objective of the four studies presented in this thesis is to identify the maternal age-associated transcriptional changes in granulosa cells of the dominant follicles during follicle development.

In the first study, mRNA expression levels of housekeeping genes were measured by real–time quantitative PCR (RT-qPCR) in granulosa cells of dominant follicles and FSH-stimulated follicles to select and validate suitable reference genes for relative gene expression analyses during maternal and follicular aging. Stability of six reference genes (GAPDH, ACTB, EIF2B2, UBE2D2, SF3A1 and RNF20) was analyzed using GeNorm, DeltaCT and NormFinder programs and comprehensive ranking order was determined based on these programs. Geometric mean of multiple genes (UBE2D2, EIF2B2, GAPDH and SF3A1) was more appropriate reference control than individual genes for the comparison of relative gene expression among dominant and FSH-stimulated follicles during maternal and/or follicular aging studies.

In the second study, maternal age-associated changes in the transcriptome of granulosa cells recovered at the time of selection of the dominant follicle from aged (n=3) and young cows (n=3) were determined by EmbryoGENE bovine oligo-microarrays (EMBV3, Agilent Technology). The mRNA expression of five transcripts (CYP19A1, PCNA, GJA1, TPM2, and VNN1*)* was confirmed in a different set of granulosa cell samples by RT-qPCR to validate microarray data. A total of 169 genes/isoforms were differentially expressed (≥ 2-fold-change; $P \leq 0.05$) in aged cows vs. young cows. These transcripts revealed inefficient 1) control of gonadotropins, and gonadotropin-induced changes in the cytoskeleton and extracellular matrix,

2) lipid metabolism and steroidogenesis 3) cell proliferation, cell cycle control and intercellular communication, and 4) higher oxidative stress responses in aged cows vs. young cows.

In the third study, changes in the transcriptome of granulosa cells of the preovulatory follicle 24 h after LH treatment from aged (n= 3) and young (n=3) were determined. A total of 1340 genes were expressed differentially (≥ 2-fold change; $P \leq 0.05$) in aged cows vs. young cows. The mRNA expression of five transcripts (RGS2, PTGS2, TNFAIP6, VNN1, NR5A2 and GADD45B) was confirmed in a different set of granulosa cell samples to validate microarray data. These transcripts were related to delayed 1) response to LH treatment 2) cellular differentiation and luteinization and 3) progesterone synthesis. Intra-follicle levels of progesterone were lower ($P < 0.05$) in aged cows compared to young and mid-aged cows.

The fourth study compared the aged-associated changes in the transcriptome of granulosa cells during follicle development from the time of dominant follicle selection to preovulatory stage (24 h after LH). In comparison to young cows, aged cows expressed fewer differentially expressed genes/isoforms (1206 vs. 2260, respectively) at ≥ 2-fold-change ($P \leq 0.05$) in the granulosa cells of the preovulatory (24 h after LH treatment) vs. the dominant follicle at selection. These transcripts in aged cows were related to late and inefficient 1) organization of cytoskeleton and cytoplasm, 2) differentiation, 3) lipid and cholesterol metabolism, 4) proliferation and 5) higher response to oxidative stress and free radical scavenging in the preovulatory follicles vs. the dominant follicle at selection. In conclusion, maternal age-alters the gene expression of granulosa cells of the dominant follicles during follicle development and results in a compromised follicular environment.

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DEDICATION

I dedicate this thesis to my wife and parents for their unconditional love and support for me.

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LIST OF ABBREVIATIONS

°C	celsius
µg	microgram
µl	microliter
µm	micro meter
18s rRNA	18S ribosomal RNA
ACTB	actin beta
ADAMTS1	disintegrin and metalloproteinase with thrombospondin motif 1
ADCY	adenylyl cyclase
AGE	advanced glycation end product
AMH	anti-mullerian hormone
ANAPC4	anaphase promoting complex subunit 4
ANNXA2	annexin A2
ARFGAP3	ADP-ribosylation factor GTPase-activating protein 3
ARPC2	actin related protein 2
Bax	B-cell lymphoma 2 associated X protein
Bcl2	B-cell lymphoma 2
BLAST	basic local alignment search tool
bp	nucleotide base pairs
BTC	betacellulin
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
BTG1	B-cell translocation gene 1
CAD	carboamoyl-phosphate synthetase 2, Aspartate transcarbamylase, dihydroorotase
CALM2	calmodulin2
CCNB1/2	cyclin B1 or B2
CCND1/2	cyclin D1 or D2
CD28	T-cell specific surface glycoprotein 28
CDK1	cyclin dependent kinase 1
CDK1/2	cyclin dependent kinase 1 or 2
CDK1B	cyclin dependent kinase 1B
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary DNA
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
cGKII	cyclic-GMP-dependent kinase II
CIDR	controlled internal drug releasing device
CLIC1	chloride intracellular channel 1
cm	centimeter
COX2	cyclooxygenase 2
CREB1	cAMP responsive element binding protein
CT	cycle threshold
CTGF	connective tissue growth factor
CV	coefficient of variation

CXCR4	C-X-C chemokine receptor type 4
Cy3	cyanine-3 green florescent dye
Cy5	cyanine-5 red florescent dye
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1
CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1
d	days
DICE	deleted in cancer 1
DNA	deoxy ribonucleic acid
DNAJA1	protein (Dnaj) homolog subfamily A, member-1
DRAK2	death associated protein kinase-related 2
EDN2	endothelin 2
EGF	epidermal growth factor
EGR1	early growth response 1
EIF2B2	eukaryotic translation initiation factor 2B, subunit 2 beta
ELMA	embryogene laboratory and microarray platform
EP2	prostaglandin E receptor 2 sub type EP2
EP4	prostaglandin E receptor 4 sub type EP4
ERK	extracellular signal-regulated kinases
ERβ	estrogen receptor beta
FADD	fas associated death domain
FDX3	ferredoxin 3
FGF2	fibroblastic growth factor 2
FGF7	fibroblast growth factor 7
FOXL2	forkhead box protein L2
FOXO3A	forkhead box protein O3A
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
FST	follistatin
g	G-force
ga or G	gauge
GADD45A/B	growth arrest and DNA-damage-inducible, alpha or beta
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GJA1	gap junction protein, alpha 1, 43kDa
GLO1/2	glyoxalase 1 or 2
GLUD	glutamate dehydrogenase
GNB2L1	guanine nucleotide binding protein
GREL	gonadal ridge epithelial like cells
GSTA1/2/4	glutathione S-transferase alpha 1 or 2 or 4
GTP	guanosine triphosphate
GV	germinal vesicle
h	hours
HAS2	hyaluronan synthase 2
hCG	human chorionic gonadotrophin
HEY1	hairy/enhancer-of-split related with YRPW motif 1
HIAP2	homolog IAP 2

HIF1α/β	hypoxia inducible factor 1, alpha or beta
HIF2α	hypoxia inducible factor 2, alpha
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HMGCS1	3-hydroxy-3-methylglutaryl coenzyme a (hmg-coa) synthase
HPRT	hypoxanthine phosphoribosyltransferase 1
HSD3β/HSD3B1	3-beta-hydroxy-delta (5)-steroid dehydrogenase
i.m	intramuscular
i.v.	intravenous
IAP	antiapoptotic protein
IDH3A	isocitric dehydrogenase subunit alpha
IGF1/2	insulin-like growth factor 1 or 2
IGF1R	insulin-like growth factor 1 receptor
IGFBP	insulin-like growth factor binding protein
IL1β	interleukin 1, beta
INHA	inhibin subunit alpha
INHBA	inhibin beta A
INSIG1	insulin induced gene 1
IU	international units
IVF	*in vitro* fertilization
JNK	c-jun N-terminal kinase
KDa	kilodalton
KITL	kit ligand
L	liters
LDH	lactate dehydrogenase
LDLR	low density lipoprotein receptor
LH	luteinizing hormone
LRP8	low density lipoprotein receptor-related protein 8
MAGOAH	mago-nashi homolog, proliferation-associated
MAPK1/14	mitogen activated protein kinase 1 or 14
MCL1	myeloid cell differentiation protein
MG	methylglyoxal
mg	milligram
MHz	megahertz
MIAME	minimal information about a microarray experiment
MIF	macrophage migration inhibitory factor
Min	minutes
mL	milliliter
mm	millimeter
MMP	matrix metallopeptidase
MOSPD3	motile sperm domain containing-3
MPF	maturation promoting factor
mRNA	messenger RNA
MRPL14	mitochondria ribosomal protein L14
MYC/B	myelocytomatosis viral oncogene homolog c or b
NCBI	national center of biotechnology
NF	normalization factor

NF-KB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
nM	nanomolar
NPC1	niemann-pick disease, type C1
NR4A1/2	nuclear receptor subfamily 4, group A, member 1 or 2
NR5A2	nuclear receptor subfamily 5, group A, member 2
NRF2	nuclear factor (erythroid-derived 2)-like 2
NRP1	neuroplin 1
PA	plasminogen activator
PACAP	pituitary adenylyl cyclase activating poly peptide
PAWR	PRKC apoptosis WT1 regulator protein
PCNA	proliferating cell nuclear antigen
PDGFA	platelet derived growth factor alpha
pg	picogram
PGE2	prostaglandin E 2
PGR	progesterone receptor
PI3K	phosphatidylinositol 3 kinase
PIGF	phosphatidylinositol glycan class F
PKA	protein kinase A
PLAT	plasminogen activator, tissue
PMSG	pregnant mare serum gonadotropin
PPARγ	peroxisome proliferative activated receptor, gamma
PRC1	polycomb responsive complex 1
PRKAA1/2	protein kinase, AMP-activated, alpha 1 catalytic subunit or alpha 2
PRL	prolactin
PRDX1	peroxiredoxin 1
PSAP	prosaposin
PTEN	phosphatase and tensin homolog deleted on chromosome 10
PTGES	prostaglandin E synthase
PTGS2	prostaglandin synthase 2
PTTG1	pituitary tumor transforming gene 1
PTX3	pentraxin 3
RAGE	receptor for advance glycation end product
RFC4	replication factor C4
RGS16	regulator of G-protein signaling 16
RGS2	regulator of G-protein signaling
RNA	ribonucleic acid
RNF20	ring finger protein 20
RPL10A	ribosomal protein 10A
RPA2	replication protein A2
RPL12	ribosomal protein L12
RPL21	ribosomal protein L21
RPL3	ribosomal protein 3
RPLO	ribosomal protein acidic
rpm	revolution per minute
RT-qPCR	real-time quantitative polymerase reaction

SAGE	serial analysis of gene expression
SAS	statistical analysis system
SCD	stearoyl-CoA desaturase
sec	seconds
SEM	standard error mean
SERPINE2	serine protease inhibitor E2
SF3A1	splicing factor 3a, subunit 1
SFRS9	splicing factor, arginine/serine rich-9
SLC22A17	solute carrier family 22, member 17
SNAP25	synaptosomal-associated protein 25
SNX9	sorting nexin 9
SOD1/2	superoxide dismutase 1 or 2
SQLE	squalene epoxidase
SREBF1/2	sterol regulatory element binding transcription factor 1
SRGN	serglycin
STAR	steroidogenic acute regulatory protein
STRA6	stimulated by retinoic acid gene-6 homolog
STRAW	staging of reproductive aging workshop
TAGE	toxic advanced glycation end product
TGFα	transforming growth factor alpha
TIMP2	tissue inhibitor metallopeptidase 2
TNFAIP6	tumor necrosis factor, alpha-induced protein 6
TNFAR	tumor necrosis factor alpha receptor
TP53	tumor protein P53
tPA	tissue plasminogen activator
TPM2	tropomyosin 2
TRIB1/2	tribbles 1 or 2
TSP2	thrombospondin 2
UBC	ubiquitin C
UBE2D2	ubiquitin-conjugating enzyme E2D 2
uPA	urokinase plasminogen activator
VASP	vasodilator-stimulated phosphoprotein
VCAN	versican
VCL	vinculin
VDAC2	volatge dependent anion channel 2
VEGF	vascular endothelial growth factor
VIPF1	vasoactive intestinal polypeptide
VNN1/2	vanin 1 or 2
vs.	versus
vol.	volume
WASF1	WAS protein family, member 1
XIAP	X-linked inhibitor of apoptosis
YWHAZ	tyrosine 3-monooxygenase activation protein, zeta polypeptide

1 CHAPTER 1: GENERAL INTRODUCTION

The phenomenon of aging is a manifestation of injurious and irreversible changes occurring to molecules, cells and organs. Among the body systems, rate of aging is much faster in reproductive organs (ovary and uterus). Consequently, pregnancy rates decline exponentially in women over 35 years of age. There are limitations to use humans as research subjects; therefore, our laboratory characterized the bovine model to understand the underlying mechanism of reproductive aging in women. Earlier studies focused on the changes in follicular and endocrine dynamics, and the oocyte competence. This thesis extends the existing model by characterizing the molecular and cellular aspects of reproductive aging.

Using the bovine specific microarrays, this study provides insight about the transcriptional changes of granulosa cells to understand the physiological changes during the follicular development in aged cows. For this purpose, we compared the granulosa cells of the dominant follicle at the time of dominant follicle selection and the preovulatory follicle stage between aged and young cows. Biological context of the transcriptional activity of the granulosa cells was explored with Ingenuity Pathway Analysis software, and the results of microarray experiments were validated by RT-qPCR.

This opening chapter of the thesis provides an overview of 1) the structure of ovarian follicles and their development, 2) functions of granulosa cells during follicular dynamics, 3) association of maternal age with subfertility in humans, mouse and cattle, 4) mechanisms of ovarian aging, and 5) endocrine and follicular dynamics of aged women and cattle.

1.1 Structure of Ovarian Follicle

Ovarian follicles are endocrine structures that are essential for female reproduction. Each follicle is composed of follicular cells (granulosa cells and theca layer) and an oocyte. Both follicular cell types are separated by a basement membrane in an antral follicle. Granulosa cells can be classified into mural and antral granulosa cells. Mural granulosa cells are adherent to the basement membrane whereas antral granulosa cells are situated towards follicular antrum. Cumulus oophorus consists of antral granulosa cells that form a hillock at one side of the follicular antrum. A layer of cumulus cells that is in immediate contact with ovum is referred as the corona radiata. Embryological origin of the granulosa cells is obscure. The granulosa cells have been proposed to originate from mesonephric cells of retti tubules or ovarii (Byskov, 1975; van den Hurk & Zhao, 2005), from ovarian blastema (Peters & Pedersen, 1967) or from mesothelium (a layer of epithelial cells from the ovarian surface epithelium) (Sawyer *et al.*, 2002; van den Hurk & Zhao, 2005). Recently, a study has identified the gonadal ridge epithelial like (GREL) cells in bovine fetal ovaries as precursor of the granulosa cells (Hummitzsch *et al.*, 2013) and ovarian surface epithelium cells (Hummitzsch *et al.*, 2013).

An Oocyte is a germ cell that is contained inside each follicle. During follicle formation, the oocyte is arrested at meiotic phase I. However, it undergoes a series of maturational changes (both nuclear and cytoplasmic) during follicular growth to achieve developmental competence (Fair, 2003). Bi-directional communication between the oocyte and somatic cells (especially cumulus cells) is necessary for the post-fertilization success of the oocyte. Surrounding cumulus cells continue to maintain a close contact with oolema until the completion of oocyte maturation (Green, 1997; Familiari *et al.*, 2006). The zona pellucida is a glycoprotein layer comprising three proteins ZP-1, -2 and -3 (Topper *et al.*, 1997) and mediates sperm-oocyte interactions as well as

participates in the zona reaction to block the polyspermy (Wassarman, 1999). The space between oolema and zona pellucida is termed as perivitelline space. This space contains a hyaluronan-rich extracellular matrix before fertilization and the cortical granules at the time of fertilization (Talbot & Dandekar, 2003). Hyaluronan is known to inhibit the fusion of somatic cell membranes (Kujawa *et al.*, 1986); however, its role in the perivitelline space is unknown and presumably related to the blocking of polyspermy (Talbot & Dandekar, 2003). In cattle, the diameter of the oocyte increases with the growth of the follicle and reaches a maximum size of 120μm (Fair, 2003). The detailed information about ultrastructure of the bovine oocyte has already been reported (Fair *et al.*, 1995; Fair *et al.*, 1997).

Information about the formation of the antrum and follicular fluid in ovarian follicles is scant. Granulosa cells have been suggested producing hyaluronan and chondroitin-sulphate-proteoglycan versican to generate an osmotic gradient and drive fluid from the theca vasculature (Clarke *et al.*, 2006; Rodgers & Irving-Rodgers, 2010). In addition to the relatively permeable nature of the follicular wall, aquaporins in granulosa cells actively transport water into the follicle (McConnell *et al.*, 2002; Rodgers & Irving-Rodgers, 2010). Consequently, multiple cavities develop within granulosa cells of preantral follicles that coalesce to form a major central cavity. The formation of antrum in a follicle is dependent on the movement of granulosa cells and remodeling of cell to cell junctions (Rodgers & Irving-Rodgers, 2010). Also, the remodeling of ovarian stroma and theca layer has been proposed as an essential feature of the expanding follicular antrum (Rodgers & Irving-Rodgers, 2010). Composition of follicular fluid is similar to the serum for low molecular weight components <100 kDa in mammals (Shalgi *et al.*, 1972; Gosden *et al.*, 1988). However, the concentration of molecules above 100 kDa is less in

follicular fluid than plasma due to the barriers existing at the level of follicular lamina or the levels of blood capillaries in theca vasculature (Zhou *et al.*, 2007).

Basement membrane is an extracellular matrix sheet that separates granulosa cells from theca layer in ovarian follicles and act as a physical barrier (Asem *et al.*, 2000) and is composed of three layers: 1) lamina lucida, 2) lamina densa and 3) lamina fibroretictularis (Bagavandoss *et al.*, 1983). Findings from several studies suggest that the granulosa cells are involved in the synthesis of the structural components of the basement membrane such as collagen type IV and fibronectin (Bagavandoss *et al.*, 1983; Rodgers *et al.*, 1995; Zhao & Luck, 1995). Growth and synthesis of the basement membrane are regulated by the granulosa cells under the stimulation of the oocyte-derived basic fibroblastic growth factor (bFGF), at the primordial follicle stage in cattle (Rodgers *et al.*, 1995). In addition, the composition of the basement membrane changes around preantral and primary stage of bovine follicular development when collagens decrease while nidogens and perlecans increase resulting in alteration in membrane permeability (Rodgers & Irving-Rodgers, 2010).

The theca layer is the outer layer of the follicle. It originates from fibroblast-like-precursor cells within cortical ovarian stroma (Orisaka *et al.*, 2006; Honda *et al.*, 2007) under the influence of theca recruitment factor (s) such as KIT-ligand (KL), bFGF, leukemia inhibiting factor (LIF) and keratinocyte growth factor (KGF) from granulosa cells (Parrott & Skinner, 2000; Young & McNeilly, 2010). The theca layer is highly vascularized and can be subdivided into outer (more fibrous) and inner (more cellular and vascular) layers. The theca layer produces androgens and provides structural support to the follicle as reviewed elsewhere (Young & McNeilly, 2010).

Summary

An ovarian follicle is composed of a single oocyte and associated follicular cells. The granulosa cells and theca cells have been proposed to evolve from ovarian surface epithelial cells and fibroblast-like stromal cells, respectively. During follicular growth, follicle develops a fluid-filled antrum, and granulosa and theca cells maintain a symbiotic relationship and are separated by a basement membrane. The oocyte is in proximity of a subpopulation of granulosa cells known as cumulus cells that play a key role in oocyte maturation. The cumulus cells penetrate the zona pellucida and establish contact with oolema until the final maturation of the oocyte. Although, the cell division of oocyte is arrested at meiotic phase-I, but it undergoes cytoplasmic and nuclear maturational changes during the follicle development.

1.2 Developmental Stages of Ovarian Follicle:

The process of follicle development is termed as folliculogenesis in which follicles grow through different stages of the development. Follicles emerge from the pool of follicles to become either an ovulatory or atretic follicles. Three major stages of follicle development are primordial follicles (resting or non-growing), preantral follicles (growing), and antral follicles.

1.2.1 Primordial Follicle

The primordial follicle is composed of a small oocyte (23-25µm) without a zona pellucida, flattened squamous granulosa cells and basement membrane. The oocyte is arrested in meiotic prophase (diplotene stage). The total number of primordial follicles (primary oocytes) varies across species (Table 1.1). The Primary oocytes of the primordial follicles originate from the primordial germ cells (PGC) that are derived from the inner cell mass of the developing blastocyst (Picton, 2001; Gordon, 2003). In cattle, after migrating to the gonadal ridge and differentiation of the ovary, PGC differentiate into oogonia by 45 days of embryonic life

(Gordon, 2003). At the end of the mitotic proliferation, oogonia resume meiosis and give rise to primary oocyte (Picton, 2001).

A primordial follicle resumes its development and enters the pool of growing follicles independent of gonadotropin support. This process is known as recruitment. During the recruitment, granulosa cells of the primordial follicles change in shape (from squamous to cuboidal) and divide slowly (Gougeon, 2010). Recruitment of the primordial follicles into the growing pool occurs either by 1) removing the inhibitory factors (Wandji *et al.*, 1996; Fortune, 2003) or 2) supplying blood borne nutrients, hormone or growth factors to the primordial follicles through vessels in ovarian medullary region (van Wezel & Rodgers, 1996) or 3) it is related to internal oocyte clock (i.e. initiation of meiosis in the oocyte during embryogenesis) (Henderson & Edwards, 1968). Activation of the primordial follicles to preantral follicle involves phosphatidylinositol 3 kinase (PI3K) signaling pathway that leads to the removal of inhibitory factors such as phosphatase and tensin homolog deleted on chromosome 10 (PTEN), forkhead box protein O3A (Foxo3A), cyclic-dependent kinase inhibitor 1B (p27) and forkhead box protein L2 (Foxl2) in oocyte (Adhikari & Liu, 2009). In addition, granulosa cells derived factor kit ligand (KITL) has been suggested to enhance activation of primordial follicle in sheep and mouse (Buratini & Price, 2011). In mice, the transition of primordial follicle into the primary follicle is considered a sensitive point for oocyte growth and mediated by the transforming growth factors (TGFβ-2, TGFβ-3, GDF-9, BMP-5 and BMP-6) (Pan *et al.*, 2005).

Table 1.1. Mean number of primordial follicles per pair of ovaries in various species**.**

Species	Primordial follicles
Cattle	210000
Rhesus Monkey	100000
Human	302000
Sheep	105450
Rodents	10,000-15000

Modified from (Gosden & Telfer, 1987; van den Hurk & Zhao, 2005)

1.2.2 Preantral Follicles

The stages of preantral follicle development are primary and secondary follicles

1.2.2.1 Primary follicle

A Primary follicle (size < 100μm) contains a single layer of one or more cuboidal granulosa cells that surround the oocyte. The granulosa cells of are mitotically active and have receptors for FSH (Bao *et al.*, 1997; Oktay *et al.*, 1997) and bFGF (Matos *et al.*, 2007). In bovine preantral follicle, activin and its receptors enhanced the proliferation of granulosa cells and growth of the oocyte in vitro (McLaughlin *et al.*, 2010). In contrast, anti-Mullerian hormone (AMH) expressed by the granulosa cells of the growing preantral follicles seems to have inhibitory action on KITL, FGF2, and FGF7 signaling to suppress the activation of primordial follicle to primary follicle in rodents and ruminants (Gigli *et al.*, 2005; Nilsson *et al.*, 2007). Oocyte of the primary follicle grows progressively and synthesizes RNA (Bachvarova, 1985). A zona pellucida is formed as an extracellular matrix coat around the oocyte (Wassarman *et al.*, 1996). Granulosa cells of the primary follicle penetrate the zona pellucida via processes to form

gap junctions with oolema (Gilula *et al.*, 1978). In cattle, the progression from the primary follicle to the secondary follicle takes around 30 days (Lussier *et al.*, 1987).

1.2.2.2 Secondary follicle

The transition of the primary follicle to a secondary follicle is marked by the addition of a second layer of granulosa cells followed by acquisition of theca layer. Thus, the secondary follicle contains 2-10 layers of cuboidal/ low columnar granulosa cells and the theca layer. In humans and cattle, granulosa cells of the secondary follicle have FSH receptors (Yamoto *et al.*, 1992; Bao *et al.*, 1997). The theca layer formation is accompanied by a vascular network (Suzuki *et al.*, 1998). Inner layer of theca cells (theca interna) expresses LH receptors and subsequently become steroidogenic (Magoffin & Weitsman, 1994). In cattle, oocyte completes its growth and attains the ability to resume meiosis around 120µm diameter (Fair *et al.*, 1995; Majerus *et al.*, 1999). However, the resumption of meiosis (i.e., break down of germinal vesicle) has been proposed to be inhibited via granulosa cells derived cyclic AMP (Sirard & Bilodeau, 1990). Oocyte development in cattle at secondary follicle stage is characterized by activation of the oocyte transcriptome, sequestration of zona pellucida, initiation of communication with granulosa cells via gap junctions, nucleolus reorganization, and synthesis of cortical granules (Fair *et al.*, 1997).

1.2.2.3 Tertiary follicle

This is the initial stage of antral follicle development.The transition of the secondary follicle into a tertiary follicle is marked by the formation of multiple small cavities in the granulosa cells. In cattle, transition from secondary to tertiary follicle take 30 days (Lussier *et al.*, 1987). At this stage, the cavity grows gradually and subsequently leads to the antrum formation (see section 1.1.2). At this stage, growth of the oocyte is characterized by intensive

mRNA and rRNA transcription, increase in oocyte volume and proliferation of oocyte organelles (Fair, 2003).

1.2.3 Antral Follicle

The antral follicles represent group of relatively large follicles characterized by a cavity (antrum) containing follicular fluid and are referred to as Graafian follicles. In cows, follicle development from the primary follicle to the antral stage (size: 0.2 mm) takes 12 weeks and afterwards 40 days to reach the preovulatory stage (Katska *et al.*, 2000; Gordon, 2003). Antral follicles are responsive to gonadotropin throughout their development until preovulatory stage of and progressively acquire steroidogenic capacity (Gordon, 2003). Additionally, antral follicles at early stage (3-4 mm) are also dependent on growth factors for development (Monniaux *et al.*, 1997). The diameter of bovine oocyte increases parallel to the diameter follicle until the follicle reaches the size of 3mm (Fair, 2010). The diameter of the oocyte plateaus at 120-130 µm while the follicle continues to grow in diameter up to 15-20 mm (Fair, 2003). At later stage of the growth period of the follicle, oocyte nucleolus turns inactive, cytoplasmic organelles migrate to the cortex and transcriptional activity becomes quiescent (Fair, 2010). Oocyte nucleus or the germinal vesicle undergoes dynamic changes during the growth phase and differentiation of the follicle. Four discrete stages of oocyte chromatin modification were identified in cattle (Lodde *et al.*, 2007; Lodde *et al.*, 2008). Germinal vesicle (GV0) is marked by filamentous chromatin in the nuclear area. The GV1 stage resembles with GV0 but is marked by few chromatin foci in the nucleus. The GV2 stage is characterized by condensation of chromatin into clumps that are distributed to nucleoplasm. At GV3 stage, chromatin is condensed into a single clump within the nuclear envelope. It has been proposed that these modifications in chromatin of oocyte are related to oocyte competence (Lodde *et al.*, 2007).

Antral follicles can be classified as healthy or atretic. Main difference between the two types is the occurrence of apoptosis in granulosa cells. Only the healthy follicle becomes the preovulatory follicle due to the progressive differentiation of granulosa cells over the time. In mono-ovular species, a single dominant follicle outgrows the cohort of antral follicles by the difference of 1 to 2 mm in diameter to achieve dominance (Sirois & Fortune, 1988; Ginther *et al.*, 1989; Ginther *et al.*, 1989; Knopf *et al.*, 1989; Adams *et al.*, 1993). This process is termed as follicle selection while remaining follicles become atretic and are termed subordinate follicles. The mechanism of selection of the dominant follicle and the difference with the subordinate follicle has been reviewed extensively in several reports (Ginther *et al.*, 1996; Mihm *et al.*, 2000; Ginther *et al.*, 2000a; Ginther *et al.*, 2000b; Fortune *et al.*, 2001; Ginther *et al.*, 2003; Mihm *et al.*, 2008; Mihm & Evans, 2008).

A surge in luteinizing hormone (LH) initiates resumption of meiosis in the oocyte and is characterized by condensation of chromosomes, progression from metaphase I to anaphase with extrusion of first polar body and arrest at metaphase II (Tsafriri *et al.*, 1972; Palma *et al.*, 2012). In xenopus, oocyte control the meiotic resumption factors such as anaphase promoting complex (APC) and cytostatic factor (CSF) through the sequential waves of polyadenylation and deadenylation in a preovulatory follicle after the LH surge (Belloc *et al.*, 2008). The breakdown of germinal vesicle and the progression of the oocyte to the subsequent stages of meiosis are facilitated by APC (Belloc *et al.*, 2008; Fair, 2010). The oocyte meiotic arrest at MII stage is facilitated by CSF (Belloc *et al.*, 2008; Fair, 2010). Also, bovine oocyte control maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) to regulate the events of oocyte maturation (Gordo *et al.*, 2001). Finally, the preovulatory follicle ruptures and extrudes the oocyte and cumulus cells by the process of ovulation. The mechanism and functions of the

granulosa cells that are associated with the development of the preovulatory follicle and are reviewed in sections 1.3 and 1.4.

Summary

Folliculogenesis resumes at the primordial follicle stage independent of gonadotropins. The primordial follicle contains oocyte of 23-25μm in diameter and is surrounded by a single layer of granulosa cells. Recruitment of the primordial follicle into the growing pool of follicles is governed by auto and paracrine factors that are secreted by the oocyte and granulosa cells. The primary follicle contains an oocyte (≤ 100μm in diameter) that is capable of synthesizing RNA rapidly, and is surrounded by single layer of mitotically active cuboidal granulosa cells. The granulosa cells contain FSH receptors and synthesize zona pellucida at this stage. Transition from the primary follicle to the secondary follicle takes 30 days in cattle. The secondary follicle contains multiple layers (less than 10) of cuboidal or low columnar granulosa cells and the theca layer. The oocyte at this stage achieves maximum diameter (120-130μm), transcriptional activity, sequestration of the zona pellucida, communication with granulosa cells, nucleolus reorganization, and synthesis of cortical granules. The granulosa cells develop LH receptors. Transition from the secondary follicle to the tertiary follicle takes additional 30 days. The size of the antral follicle is about 0.2-0.4 mm in the beginning and progressively increases to 3-4mm size under the influence of growth factors independent of gonadotropins. Later, the development of the dominant follicle until the preovulatory follicle is dependant on gonadotropin and involves increased steroidogenesis. The oocyte at this stage does not increase in diameter and passes through GV0 to GV3 stages. The antral follicle can be categorized as healthy and atretic and follows the well characterized ovarian follicular dynamics.

1.3 Follicular and Endocrine Dynamics in Cattle

Average duration of bovine estrous cycle is reported to be 21-days (Chapman, 1937; Adams *et al.*, 2008) however, the duration of two waves estrous cycle is 20 days whereas in three waves cycle is 23 days long (Ginther *et al.*, 1989; Fortune, 1993; Noseir, 2003). In general, estrous cycleconsists of four phases i.e. proestrus (2-3 days), estrus (1-2 days), metestrus (3-5 days) and diestrus (14-15 days) (Forde *et al.*, 2011) or divided into follicular phase (before ovulation) and luteal phase (after ovulation) (Chapman, 1937) . Proestrus is characterized by regression of CL, maturation of a preovulatory follicle, decrease in serum progesterone concentrations, increase in serum estradiol concentrations and LH pulse frequency (Forde *et al.*, 2011). Estrus is characterized by signs of sexual receptivity, increased vaginal and cervical mucus secretions, very low serum progesterone concentrations, decreasing estradiol concentrations and induction of LH surge (Roelofs *et al.*, 2010) The duration of standing estrus varies from 8-18 h in different breeds of cattle (Forde *et al.*, 2011). Metestrus is characterized by ovulation about 10-15 h after the end of estrus, the development of CL, and a gradual increase in plasma progesterone concentrations (Forde *et al.*, 2011). In early diestrus, the growth of CL is rapid and plasma concentrations of progesterone increase linearly but as the diestrus progresses, the growth of the CL and plasma concentrations of progesterone reach a plateau (Forde *et al.*, 2011).

Rajakoski first described the wave-like pattern of follicular growth of bovine estrous cycle (Rajakoski, 1960) and this theory was later confirmed through monitoring of ovarian follicles by ultrasonography (Pierson & Ginther, 1984; Knopf *et al.*, 1989; Griffin & Ginther, 1992). Bovine estrous cycle is composed of two or three waves and first follicle wave in both wave patterns emerges on the day of ovulation (Day 0) (Ginther *et al.*, 1989). In 2-wave cycle,

the second wave emerges on Day 9-10 whereas in 3-wave cycle; second wave emerges on Day 8-9 followed by a third wave on Day 15-16 (Ginther *et al.*, 1989). Follicle wave emergence is characterized by a preceding rise in FSH and a concurrent growth of 7-11 follicles of 4 mm in diameter (Adams *et al.*, 1992). A selection of the dominant follicle occurs when FSH levels are declining (Adams *et al.*, 1992; Adams *et al.*, 1993; Ginther *et al.*, 1996; Mihm *et al.*, 1997; Ginther, 2000; Mihm & Evans, 2008). After the selection, both the dominant and subordinate follicles undergo three distinct phases of development i.e. growing, static and regressing and these phases indicate the functional status of the follicle (Singh *et al.*, 1998; Singh & Adams, 2000). In 3-wave cycle, the dominant follicles from first and second wave regress due to high progesterone concentrations and decreased LH pulse frequency during the diestrus phase (Ginther *et al.*, 1989). In both 2-wave and 3-wave cycles, a last wave yields an ovulatory follicle under the declining progesterone concentrations and high concentrations of estradiol from the dominant follicle (Ginther *et al.*, 1989; Adams *et al.*, 2008). Consequently, LH pulse frequency and amplitude increase and LH surge occur to cause ovulation (Adams *et al.*, 2008). It has been reported that the length of ovulatory wave is shorter by 3-days in two wave cycles than three wave cycles; however, no significant difference in pregnancy rates has been reported (Bleach *et al.*, 2004; Adams *et al.*, 2008; Dias *et al.*, 2011).

Summary

In cattle, estrous cycle is composed of two or three follicular waves. The duration of estrous cycle in two wave animals is 20 days whereas three wave animals have the duration of 23 days (Ginther et al., 1989; Fortune, 1993; Noseir, 2003). Estrous cycle is marked by four stages based on the follicular and endocrine dynamics. Concept of ovarian follicular waves per estrous cycle was introduced by Rajakoski in 1960 and validated by using ultrasonography of ovarian

follicles (Ginther et al. 1984). Temporal association of FSH with emergence of follicular wave and the selection of the dominant follicle were established in cattle (Adams et al. 1992). Temporal relationship of LH with ovulation was established in cattle. Pregnancy rates between the cows having either 2 or 3 follicular waves were not statistically different.

1.4 Granulosa Cell Function during Follicular Wave Development

1.4.1 Granulosa Cell Products and Receptors at Emergence of Follicular Wave

In humans and cattle, the emergence of follicular wave is defined as a sudden appearance of a group of 2-5 mm antral follicles (Adams *et al.*, 1992; Baerwald *et al.*, 2003; Baerwald *et al.*, 2012). This sudden appearance of the follicles has been associated with temporal fluctuation of FSH concentrations in cattle and humans (Adams *et al.*, 1992; Baerwald *et al.*, 2003; Baerwald *et al.*, 2012). The expression of FSH receptors on the granulosa cells of antral follicle is considered a major factor for early survival of the antral follicle (Chun *et al.*, 1996; Webb *et al.*, 1999). However, what leads to the expression of FSH receptors in the granulosa cells remains unknown. One theory suggests that the production of androgens and expression of their receptors in granulosa cells of early antral follicles may lead to the development of the FSH receptors (Tetsuka & Hillier, 1996; Tetsuka & Hillier, 1997). At the same time autocrine or paracrine factors such as EGF, IGF-1 and IGF-2, FGF and transforming growth factor alpha (TGFα), enhance the granulosa cell proliferation and survival (Monniaux *et al.*, 1997). In addition, granulosa cells of newly emerged follicle start expressing mRNA of P450scc and P450aromatase for cholesterol and estradiol synthesis (Webb *et al.*, 1999).

1.4.2 Granulosa Cell Products and Receptors in the Dominant Follicle at the time of Selection

The selection of the bovine dominant follicle occurs when plasma FSH levels are declining (Adams *et al.*, 1992) and dominant follicle is dependent on LH pulses for further growth (Mihm & Austin, 2002; Mihm & Evans, 2008). However, a selected dominant follicle requires basal FSH after deviating in growth rate from the next largest follicle (Ginther *et al.*, 1999; Ginther *et al.*, 2000a). In this section, autocrine and paracrine factors that are secreted by the granulosa cells of dominant follicle at the time of selection are highlighted.

Inhibins and Follistatin: Inhibins are dimeric glycoproteins with alpha and beta subunits, and they exist in bovine follicular fluid as homo- or hetero-dimeric forms (Robertson et al., 1987). Granulosa cells secrete inhibins and express genes for both subunits of inhibins. Similarly, follistatin is produced by the granulosa cells (Singh & Adams, 1998) and suppresses FSH through neutralization of activins (Nakamura et al., 1990). Release of inhibins from granulosa cells of the selected dominant follicle and the recruited cohort of follicles suppress FSH in mono ovular species (Kaneko et al., 1997; Bleach et al., 2001). As a result, FSH support to the growing cohort of follicles is withdrawn while the dominant follicle continues to grow due to its utilization of LH. In addition, inhibins are known to modulate the follicle growth and steroidogenesis in vivo and *in vitro* (Mihm & Austin, 2002). The bovine estrogenic follicles contain an increased amount of the dimeric inhibins over160 KDa in size while non estrogenic or atretic follicles contain inhibins of 32-34 KDa in size. (Ireland et al., 1994). A significant increase in high molecular weight inhibins has been observed during and after the selection of the dominant follicle in heifers (Austin et al., 2001). These findings suggest that inhibins suppress FSH secretions.

Follistatin is a glycosylated protein with six isoforms (size range: 31-39 KDa) (Esch *et al.*, 1987). In cattle, expression of mRNA of follistatin increases with the diameter of dominant follicle (Shukovski et al., 1992). The granulosa cells of the dominant follicle show intense immunostaining during the growing, early static and preovulatory phases (Singh & Adams, 1998). Intrafollicular concentrations of activin A and follistatin did not differ between the future dominant follicle and the largest subordinate follicle on Day 3 after ovulation in cattle (Mihm et al., 2000). However, immunohistochemical analysis of the granulosa cells from the dominant follicle at Day 3 of first follicular wave showed higher absorptive index for follistatin as compared to the largest subordinate follicle (Singh & Adams, 1998). This indicates that follistatin may play a role in the selection of the dominant follicle. A temporal association between intrafollicular estradiol concentrations and the amount of immunostained follistatin in the dominant follicle suggests the role of follistatin in the selection of dominant follicle (Singh & Adams, 1998; Singh *et al.*, 1998).

Insulin-like growth factors (IGF) and binding proteins (IGFBPs): IGF-1 and its receptors are expressed by granulosa cells in cattle (Stewart *et al.*, 1996; Armstrong *et al.*, 2000), sheep (Hastie & Haresign, 2006) and humans (Zhou et al., 1991). At the time of the dominant follicle selection, IGFs increase the responsiveness of the dominant follicle to the gonadotropins in cattle, sheep and pigs (Matsuda et al., 2012). IGF-1 also increases the estradiol production of granulosa cells from large follicles (>8 mm) (Spicer *et al.*, 1993) and ensures follicular survival by suppressing the apoptosis (Matsuda et al., 2012).

Insulin-like growth factor binding proteins (IGFBPs) inhibit IGF interaction with its receptors (Clemmons *et al.*, 1992; Clemmons, 1997; Clemmons, 1998; Clemmons *et al.*, 1998). In response to FSH, IGFBP proteases are produced by the dominant follicle. In cattle, IGFBP

proteases increase the bioavailability of IGFs by degrading the IGFBP (Mazerbourg *et al.*, 2000). Increased IGFs synergizes with FSH and lead to the functional selection of the dominant follicle (Fortune *et al.*, 2001). Intrafollicular concentrations of IGFBP-2, -4, and -5 decrease gradually with the growth of antral follicles whereas IGFBP < 40 KDa increase in follicles with atresia (Echternkamp *et al.*, 1994; Monniaux *et al.*, 1997). In cattle, IGFBP-2 is expressed by granulosa cells while IGFBP-4 and -5 are expressed by theca cells (Armstrong *et al.*, 1998). In cattle, reduction in intrafollicular concentrations of IGFBP-2 and -4 by IGFBP proteases and synergestic effect of IGFs and FSH leads to increase in estradiol production and growth rate in the dominant follicle (morphological selection) (Armstrong *et al.*, 1998; Mihm *et al.*, 2000; Fortune *et al.*, 2001; Webb *et al.*, 2007).

Estradiol: Increased synthesis of estradiol ensures the morphological selection of the dominant follicle (Bodensteiner *et al.*, 1996; Fortune *et al.*, 2001). Intrafollicular concentration of estradiol is a reliable marker to identify the future dominant follicle among the cohort of 5-8.5 mm size bovine follicles (Mihm *et al.*, 2000). In cattle, intrafollicular concentration of estradiol differ markedly at the time of selection (on Day 3 of follicular wave) between the dominant follicle and the largest subordinate follicle (Singh *et al.*, 1998; Ginther *et al.*, 2000b). Secretion of estradiol by the dominant follicle coincides with the decline in FSH (Ginther *et al.*, 2000a; Fortune *et al.*, 2001). In bovine granulosa cells, mRNA levels for the aromatase enzyme (catalyze the conversion of androgens to estradiol) increase during the initial two days of follicular wave (Xu *et al.*, 1995). Consequently, the dominant follicle on the fourth day of follicular wave has a higher concentration of estradiol compared to the recruited follicle during the initial two days after the follicular wave emergence. Also, mRNA levels of P450scc (catalyze the conversion of cholesterol to pregnenolone) had been reported higher in granulosa cells of the

selected dominant follicle (Day 4 of wave) than recruited follicles obtained before selection (Xu *et al.*, 1995).

Luteinizing hormone receptors (LHR): In cattle, the acquisition of LHR by granulosa cells of a single follicle has been associated with selection of the dominant follicle (Ginther *et al.*, 1996; Bao *et al.*, 1997; Liu *et al.*, 2009). Granulosa cells of the newly selected dominant follicle show an increased expression of LHR during their early growth phase and acquire the enhanced ability to bind LH (Beg *et al.*, 2001; Mihm & Evans, 2008). In heifers, presence of LHR in granulosa cells of the future dominant follicle has been detected 8 h before the time of follicle deviation (Ginther *et al.*, 2001). These findings support the hypothesis that survival of the dominant follicle under low level of FSH is due to the acquisition of LHR in granulosa cells; whereby the dominant follicle at selection is responsive to the pulses of LH (Ginther *et al.*, 1996; Bao *et al.*, 1997; Liu *et al.*, 2009). However, findings from few studies (Bodensteiner *et al.*, 1996; Evans & Fortune, 1997) contest this hypothesis and suggest that acquisition of the LHR by granulosa cells of the dominant follicle occurs after the selection rather than before selection; therefore, increase in estradiol is related to the selection of the dominant follicle rather than the increase in LHR receptors.

1.4.3 Candidate Genes for the Dominant Follicle Selection

Using SAGE (Serial Analysis of Gene Expression), several genes have been identified in bovine granulosa cells which are regulated at the time of dominant follicle selection (Mihm *et al.*, 2008). Comparison of mRNA of granulosa cells between cohort of follicle and future dominant follicle (Mihm *et al.*, 2008) revealed up-regulation of the transcripts related to 1) intracellular signaling such as annexin A2 (ANNXA2), calmodulin-2 (CALM2), chloride intracellular channel 1 (CLIC1), and stimulated by retinoic acid gene-6 homolog (STRA6), 2)

cellular proliferation such as cylin D2 (CCND2), 3) estradiol synthesis such as cytochrome P450 aroma (CYP19A1), 4) anti-oxidant activity such as glutathione S-transferase alpha-2 (GSTA2), 5) LH responsiveness such as luteinizing hormone receptors (LHR), 5) anti-apoptotic activity such as growth arrest and DNA damage-inducible beta (GADD45B) by antagonizing the tumor necrosis factor alpha receptor (TNFAR) induced apoptosis and proapoptotic c-Jun N-terminal kinase (JNK) signaling (De Smaele *et al.*, 2001), macrophage migration inhibitory factor (MIF), and replication factor C4 (RFC4), 6) RNA splicing such as splicing factor, arginine/serine rich-9 (SFRS9), 7) cell structure such as actin beta (ACTB), motile sperm domain containing-3 (MOSPD3), 8) protein transport such as sorting nexin 9 (SNX9), mago-nashi homolog, proliferation-associated (MAGOAH), protein (Dnaj) homolog subfamily A, member-1 (DNAJA1), solute carrier family 22, member 17 (SLC22A17) and 9) lipid synthesis such as stearoyl-coA desaturase (SCD). In addition, genes related to extracellular matrix regulation such as serine protease inhibitor E2 (SERPINE2), inhibin subunit alpha (INHA) were up-regulated in the dominant follicle compared with cohort of follicles and corroborate with the conclusion of the previous studies (Evans *et al.*, 2004; Fayad *et al.*, 2004).

Comparison of bovine granulosa cells of the dominant follicle to those from cohort of follicle or the subordinate follicle showed that the mRNA expression of 14 genes was down-regulated (Mihm *et al.*, 2008). These genes were related to secretion such as serglycin (SRGN), protein synthesis such as ribosomal proteins (RPL21, RPL10A, RPL12, RPL3), G-protein signaling such as guanine nucleotide binding protein (GNB2L1), proliferation such as anaphase promoting complex subunit 4 (ANAPC4), nitrogen metabolism such as glutamate dehydrogenase (GLUD1), and lipid synthesis, transport and metabolism such as prosaposin; (PSAP), and

prostaglandin-E synthase (PTGES). However, the exact function of these genes still requires further studies.

1.4.4 Granulosa cell Function during Follicle Atresia and Apoptosis

Estradiol: Depending on the phase of bovine estrous cycle, the dominant follicle either regresses (in the luteal phase of the cycle) or continues to differentiate after the selection until ovulation (in the follicular phase of the cycle). Intrafollicular estradiol concentration has been shown to reduce in follicles collected during early to late-static phase of dominant follicle development in comparison to the time of dominant follicle selection (Singh *et al.*, 1998). Apoptosis in granulosa cells is marked by DNA fragmentation. In cattle, apoptosis in granulosa cells has been negatively associated with intrafollicle levels of estradiol (Jolly *et al.*, 1994). Loss of estradiol production in bovine granulosa cells serves as a marker for the follicles that are destined for atresia (Bellin & Ax, 1984; Austin *et al.*, 2001; Evans *et al.*, 2004). Androgens have been reported promoting apoptosis while estradiol has been suggested impeding apoptosis and enhancing the proliferation of granulosa cells (Billig *et al.*, 1993; Quirk *et al.*, 2004). Granulosa cells of atretic follicles also express decreased estrogen receptors (Richards, 1975). In cattle, decrease in estradiol production during atresia of the dominant follicle is not due to deficiency of androgen substrate from theca cells for aromatization but may be due to decreased activity of aromatase enzyme in granulosa cell (Valdez *et al.*, 2005).

Fas ligand and growth/survival factors: Fas (antigen, a cell surface receptor) and its ligand (FasL) initiate apoptosis in bovine granulosa cells during follicular development (Porter *et al.*, 2000; Porter *et al.*, 2001). In cattle, mRNA levels of Fas and FasL increased in granulosa cells of the atretic or subordinate follicles compared to healthy dominant follicles (Porter *et al.*, 2001). Also, only the granulosa cells of bovine dominant follicles before LH surge are

susceptible to the Fas-induced apoptosis (Porter *et al.*, 2001). It has been hypothesized that lack of appropriate growth or survival factors (IGF, bFGF, EGF) and suppression of anti-apoptotic proteins (IAPs; Xiap and Hiap 2) may lead to the expression of Fas and FasL on granulosa cells of atretic or subordinate follicles *in vitro* (Hsu & Hsueh, 1997; Li *et al.*, 1998; Matsuda *et al.*, 2012).

Intrinsic and extrinsic molecular mechanisms of apoptosis are reviewd elsewhere (Elmore, 2007) and may be involved in apoptosis of the granulosa cells in atrertic follicle (Fig. 1.1). Intrinsic mechanism involves the activation of TP53 transcription factor in response to decreased sensitivity to gonadotropin (Tilly *et al.*, 1995; Tilly *et al.*, 1995b) due to reduced intrafollicular concentrations of growth factors EGF, IGF, FGF (Quirk *et al.*, 2000; Quirk *et al.*, 2004) and estradiol in a subordinate follicle (Quirk *et al.*, 2006). As a result, TP53 blocks the antiapoptotic genes expression such as bcl2 family (Tilly *et al.*, 1995b) and increases the expression of FasL or translocates Fas to plasma membrane from Golgi apparatus (Kim *et al.*, 1999). Decreased intrafollicular concentrations of growth factors also induce the expression of phosphatidylserine from inner plasma membrane to outer plasma membrane and mark the granulosa cells for apoptosis (Quirk *et al.*, 2000). Extrinsic mechanism overlaps the intrinsic mechanism. Upon binding with their receptors, FasL and tumor necrosis factor alpha (TNFα) induce expression of Fas associated death domain (FADD) in tumor necrosis factor receptors family (Fas and TNFAR) (Manabe *et al.*, 2004). FADD activates phosphatidylserine directly or indirectly via caspases (Manabe *et al.*, 2004). Caspases induce capase activated-DNase (CAD) to cause fragmentation of the DNA of the cells and apoptosis (Boone & Tsang, 1998).

1.4.5 Candidate Genes in Apoptosis and Atresia

It appears that the dominant follicle survives against apoptosis by expressing antiapoptotic genes and producing a high amount of estradiol. In microarray based study, decreased secretion of estradiol by granulosa cells of the largest subordinate follicle has been related to higher expression of apoptotic genes such as betaglycan, death associated protein kinase-related 2(DRAK2), TNFα, carboamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase proteins encoding gene (CAD) and prostaglandin synthase 1 (PTGS1) (Evans *et al.*, 2004). In contrast, granulosa cells of the dominant follicle expressed enhanced expression of genes related to inhibition of apoptotic pathway i.e. CYP19A1, LHR, estradiol receptor beta; ERβ, deleted in cancer 1(DICE), and myeloid cell differentiation protein (MCL-1) (Evans *et al.*, 2004).

In a study, two novel genes i.e. voltage-dependent anion channel 2 (VDAC2) and mitochondrial ribosomal protein L41(MRPL41) were identified in granulosa cells of the dominant follicle compared to those from the subordinate follicle using bovine cDNA microarrays (Zielak *et al.*, 2007). These genes were related to the inhibition of apoptosis and binding with anti-apoptotic factor Bcl-2 (Zielak *et al.*, 2007). The global gene expression profiling of bovine granulosa cells and theca layer of the subordinate follicle (Hayashi *et al.*, 2010) revealed high levels of mRNA for IGFBP-5, growth arrest and DNA damage inducible 45 A (GADD45A), thrombospondin 2 (TSP2) and lower mRNA levels of CYP19A1, FSHR, glutathione peroxidase 3 (GPX3), tribbles homolog (TRIB2), SCD, and phosphatidylinositol glycan, class F (PIGF) as compared with the dominant follicles. These results indicate that the apoptosis in the granulosa cells of the subordinate follicles is induced by certain genes (Hayashi *et al.*, 2010).

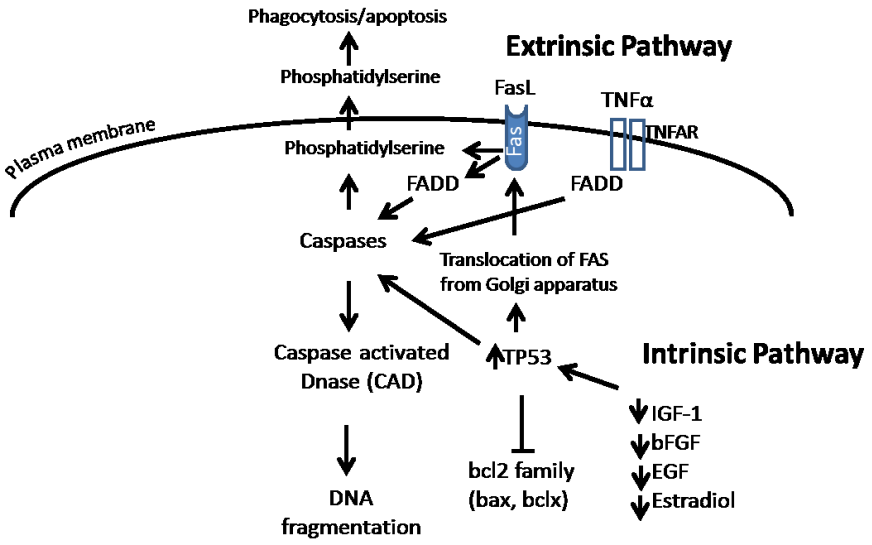

Figure 1.1 Intrinsic and extrinsic mechanisms of apoptosis in granulosa cells of an atretic follicle. Intrinsic mechanism involves activation of TP53 transcription factor in response to decrease sensitivity of granulosa cells to gonadotropin due to decreased intrafollicular concentration of growth factors. Extrinsic mechanism involves binding of Fas and TNFα to their receptors. Both pathways overlap and lead to the induction of phosphatidylserine and caspases to induce apoptosis in granulosa cells of the atretic follicle. (Illustration is based on literature review in section 1.4.4).

1.4.6 Granulosa cell Function in Dominant Follicle before LH Surge

1.4.6.1 Steroidogenesis

During the dominant phase of the follicle (from Day 3 to Day 6 of first follicular wave), intrafollicular ratio of estradiol to progesterone decreases suggesting increased synthesis of estradiol by bovine granulosa cells (Singh *et al.*, 1998). The expression of aromatase is higher in mural granulosa cells than antral granulosa cells while cumulus cells do not express aromatase (Turner *et al.*, 2002). According to the two-cells, two-gonadotropin theory, FSH stimulates aromatase activity (Armstrong, 1979) and aromatase expression is regulated by FSH in

conjunction with estradiol to establish a fully differentiated healthy preovulatory follicle as demonstrated by estrogen receptor beta knockout mouse model (Couse *et al.*, 2005). FSH also induces LH receptors on granulosa cells of the dominant follicle and in turn aromatase activity is regulated by LH in the preovulatory follicle (Hillier *et al.*, 1994). Growth factors such as EGF, and prolactin (PRL), and steroids such as progesterone and dexamethasone inhibit the activity of aromatase in cultured rat granulosa cells (Hsueh & Erickson, 1978; Adashi & Reshick, 1986; Fortune *et al.*, 1986; Mendelson *et al.*, 1986). In rat preovulatory follicle, granulosa cells are capable of producing progesterone after the acquisition of LH receptors and such evidence also existed in bovine preovulatory follicle (Fortune & Quirk, 1988). Consequently, bovine granulosa cells not only aromatize androgens but also provide pregnenolone to theca cells for increased androgen production (a substrate for estradiol synthesis) (Fortune & Quirk, 1988). In addition, estradiol exert a negative feedback to granulosa cells to limit the conversion of pregnenolone to progesterone (Fortune & Hansel, 1979). These findings suggest that the granulosa cells of the dominant follicle are actively involved in steroidogenesis (especially the estradiol) before LH surge.

1.4.6.2 Proliferation/cell cycle

Granulosa cells of the preovulatory follicle divide rapidly before ovulatory surge of LH whereas LH surge blocks the progression of granulosa cell cycle and proliferation. The pattern of granulosa cell proliferation is well studied in hypophysectomized rat models (Richards, 1980; Robker & Richards, 1998). Binding of the cyclins D2 and E with respective cyclin dependent kinases (cdk -4 or -6 and -2) result in cell cycle progression through G1 to S phase, respectively (Xiong *et al.*, 1992) while inactivation of the cdks by CDKN1B or $p27^{Kip1}$ block cell cycle progression through G1 phase (Polyak *et al.*, 1994).

Proliferation of rat granulosa cells is regulated by pituitary gonadotropins (FSH and LH), estradiol (Robker & Richards, 1998), IGF (Zhou *et al.*, 1995) and activins (Miro & Hillier, 1996). Gonadotropins (FSH/LH) accelerate proliferation of the granulosa cells by modulating the cyclic adenosine monophosphate (cAMP) levels and inducing protein kinase-A (PKA) pathways (Jonassen & Richards, 1980; Richards & Kirchick, 1984). However, cAMP levels alter in response to high or low doses of gonadotropins and result in modulation of the activity of cell cycle activators (cyclin D2 and cyclin E2) and inhibitors (cyclin-dependent kinase inhibitor 1B; CDKN1B) (Robker & Richards, 1998; Robker & Richards, 1998). In addition, the gonadotropins exert its effect on different levels of cell cycle machinery, i.e. either at G1-S phase or M-G1 phase (Robker & Richards, 1998). Although, both FSH and Estradiol induce the expression of cyclin D2 and cyclin E in granulosa cells of rodents, but estradiol is considered more mitogenic as it concurrently represses the expression of CDKN1B (Robker & Richards, 1998) to allow the cell cycle progress.

The rapid phase of growth after acquiring responsiveness to gonadotropin is characterized by increased labeling of granulosa cells by titrated thymidine in rat (Hirshfield, 1986) and 5-bromodeoxyuridine (BrdU) (Gaytan *et al.*, 1996) and the increased expression of proliferating cell nuclear antigen (PCNA) protein and mRNA (Oktay *et al.*, 1995). Analysis of the bovine granulosa cell transcripts from the periovulatory follicle (2-h before LH) also revealed the genes associated with cell division, proliferation and cellular development (Gilbert *et al.*, 2011). However, at later stage of the dominant follicle growth, granulosa cells progressively lose the ability of proliferation and acquire high steroidogenic activity (Clement *et al.*, 1997).

1.4.7 Granulosa cell Function in Preovulatory Follicle after LH Surge

Increased levels of estradiol (above threshold) positively affect GnRH pulse generator (Wiltbank *et al.*, 2002; Aerts & Bols, 2010) and increased LH pulse frequency which culminates in LH surge In turn, LH surge initiates a cascade of events in granulosa cells to ensure the ovulation. The events occurring in granulosa cells in response to LH surge are summarized in the following sections (1.4.7.1, and 1.4.7.2)

1.4.7.1 Differentiation and luteinization

A surge of luteinizing hormone blocks the progression of granulosa cell cycle and proliferation. Mechanisms by which LH surge terminates granulosa cell proliferation involve 1) rapid inhibition of cyclin D2 transcription followed by inhibition of cyclin E and 2) increase in levels of cdk inhibitors (CDKN1B, Cyclin dependent kinase inhibitor-1; CK1, cyclin dependent kinase inhibitor-1C; CDKN1C) (Robker & Richards, 1998). This marks the terminal differentiation of the granulosa cells into the luteal phenotype (Richards, 1994).

Following LH surge, steroidogenic capabilities of granulosa cells change dramatically in cattle. Progesterone concentrations in follicular fluid increase whereas estradiol and androgen concentration decline (Hansen *et al.*, 1988). Progesterone biosynthesis involves two enzymatic steps: 1) conversion of cholesterol into pregnenolone by P450 side chain cleavage enzyme (P45scc), and 2) conversion of pregnenolone into progesterone by 3βHSD. A transient decrease in mRNA levels of P450scc (up to 96%) and of 3βHSD by 4.2-folds has been observed in bovine granulosa cells after LH surge than before LH surge (Voss & Fortune, 1993). However, both P450scc and 3βHSD mRNA levels increased in later stages after LH surge and in corpus luteum of rats and cattle (Rodgers *et al.*, 1987; Hickey *et al.*, 1990). Steroidogenic acute regulatory protein (STAR) which transport cholesterol from outer to inner mitochondrial membrane, is

absent in granulosa cells before LH surge but STAR mRNA levels increase temporally in cattle (Gilbert *et al.*, 2011) and in human granulosa cells after LH surge (Christenson & Strauss, 2001). Increase in progesterone concentration, in bovine follicular fluid, indicates luteinization of granulosa cells and theca layer (Voss & Fortune, 1993). A biphasic pattern in increase of intrafollicle concentration of progesterone occurs after 1.5 h and 23 h of LH surge in cattle (Fortune *et al.*, 2009). These findings suggest that the granulosa cells markedly shift from estradiol production to progesterone production after LH surge.

1.4.7.2 Ovulation

Morphological changes within the preovulatory follicle after LH surge has been reviewed elsewhere e.g. (Smith *et al.*, 1994; Niswender *et al.*, 2000). Putative biochemical substances involved in ovulation are progesterone, prostaglandin and proteases.

Progesterone and ovulation: After the LH surge, biosynthesis of progesterone is initiated in bovine and murine granulosa cells of the mature follicle concomitant with induction and up-regulation of the mRNA of progesterone receptors (PGR) (Fortune & Quirk, 1988; Conneely *et al.*, 2002). Mice lacking PGR fail to ovulate (Conneely *et al.*, 2002). In addition, progesterone induces transcription of disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-1) (Espey *et al.*, 2000; Robker *et al.*, 2000). ADAMTS-1 cleaves Versican (VCAN) to expand cumulus oocyte complex and assist ovulation in mouse (Curry, 2010) and blocking of ADAMTS-1 results in inhibition of oocyte release in rat (Espey *et al.*, 2000). In addition, progesterone induces the expression of Cathepsin L (catL) that helps in degradation of the follicular wall (Sriraman & Richards, 2004). Progesterone via PGR also transiently induces expression of pituitary adenylyl cyclase activating poly peptide (PACAP) in rat granulosa cells

(Ko & Park-Sarge, 2000; Park *et al.*, 2000) to regulate acute progesterone production and luteinization (Gras *et al.*, 2005).

Genes that are regulated by the LH-mediated PGR in rat and mice mural granulosa cells include hypoxia inducible factors (e.g. HIF-1α, HIF-2α and HIF-1β) (Kim *et al.*, 2009), endothelin-2 (EDN2) (Palanisamy *et al.*, 2006), synaptosomal-associated protein 25 (SNAP25) (Shimada *et al.*, 2007), cyclic-GMP-dependent kinase II (cGK II) (Sriraman *et al.*, 2006), peroxisome proliferator-activated receptor gamma (PPARγ) (Kim *et al.*, 2008), vascular endothelial growth factor (VEGF) (Fraser *et al.*, 2005; Kim *et al.*, 2009), and C-X-C chemokine receptor type 4 (CXCR4) (Kim *et al.*, 2009). All of these genes are involved in the degradation of follicular wall, vasodilation and vascular permeability, vascular dynamics and inflammation (Kim *et al.*, 2009).

Prostaglandins and ovulation: In response to LH surge, prostaglandins (PGF2 and PGE2) are synthesized from arachidonic acid in rat granulosa cells (Clark *et al.*, 1978; Richards, 2006). The treatment of laboratory animals, cattle and marewith non-steroidal anti-inflammatory agents or indomethacin inhibit ovulation (Armstrong & Grinwich, 1972; De Silva & Reeves, 1985; Cuervo-Arango & Domingo-Ortiz, 2011). This leads to the hypothesis that the ovulation is an inflammatory process (Espey, 1980). Prostaglandin synthase or cyclooxygenase (metabolize arachidonic acid into active prostaglandin) mRNA is induced rapidly but transiently within 4 h after LH or human chorionic gonadotropin (hCG) treatment of mice granulosa cells (Joyce *et al.*, 2001). The mRNA expression of prostaglandin synthase 2 (PTGS2) is decreased by 6 h, but its protein persists even 20-24 h later (Joyce *et al.*, 2001). In conjunction with LH, PTGS2 mRNA and protein in granulosa cells is regulated by EGF-like factors (amphiregulin, epiregulin and betacellulin) and hyaluronan synthase-2 (HAS-2) in rat and cattle cumulus cells or

granulosa cells (Kimura *et al.*, 2002; Ochsner *et al.*, 2003; Schoenfelder & Einspanier, 2003; Park *et al.*, 2004). Considering the consistent duration (~10 h) from PTGS2 induction to ovulation in rats, mares and cows, PTGS2 is considered as an ovulation indicator (Richards, 1997).

In follicular cells of rodents, prostaglandins activate proteolytic enzymes, stimulate angiogenesis (Sakurai *et al.*, 2003), and promote the nitric oxide synthase activity in rabbits (Yamauchi *et al.*, 1997). The presence of G-coupled prostaglandin receptors (EP2 and EP4) in mice granulosa cells within 3 h after hCG administration suggest an active role of prostaglandin during the ovulation (Segi *et al.*, 2003). PGE2 temporally induces the expression of TNFAIP6 in mice, which in turn stabilizes the extracellular matrix (Fulop *et al.*, 2003). PGE2 also regulates the expression of interleukin-1β (IL1β) (Chung *et al.*, 2000), a cytokine and capable of inducing ovulation and oocyte maturation (Takehara *et al.*, 1994). It has been suggested that prostaglandins and progesterone are two distinct molecules that act via separate genetic program, but finally converge on cumulus oocyte complex for matrix regulation (Richards, 2006).

Proteases and ovulation: The proteolytic nature of the proteases provides a necessary impetus for follicular rupture and matrix remodeling after LH surge (Murdoch & Gottsch, 2003). Plasminogen activators or plasmins (PA) and matrix metalloproteinases (MMPs) are two major families of enzymes that govern tissue degradation and remodeling. Plasminogen activators (PA) have two subtypes: urokinase (uPA) and tissue plasminogen activators (tPA) (Murdoch & Gottsch, 2003). Both are secreted either from murine granulosa cells or by the ovarian surface epithelial cell (Richards, 2006). In response to LH surge, the mRNA expression of PA and MMPs upregulates in bovine granulosa cells (Fortune *et al.*, 2009). MMPs are endopeptidases that degrade collagens, elastin, proteoglycans and adhesion molecules (Curry &

Osteen, 2001; Curry & Osteen, 2003; Curry & Smith, 2006). An enzyme theory of ovulation involving proteases, LH, ovarian cells, plasminogen activators, collagenases and TNFα has been proposed for the degeneration of the follicular wall during ovulatory process (Murdoch & Gottsch, 2003).

The role of ADAMTS in bovine ovulatory follicle has been reviewed elsewhere (Fortune *et al.*, 2009). ADAMTS either bind extracellular matrix components or attach to the cell surface (Tang, 2001). In response to LH, ADAMTS are readily secreted by the bovine granulosa cells and theca interna of the ovulatory follicle and are suggested having both time and cell specific expression pattern (Madan *et al.*, 2003; Fortune *et al.*, 2009). In bovine granulosa cells, the mRNA expression of ADAMTS-1, -2, -5 and -9 increases by progesterone, PGR and prostaglandins (Fortune *et al.*, 2009). These findings indicate a complex but desired role of proteases in ovulation (Fortune *et al.*, 2009).

1.4.8 Genes involved in Differentiation, Luteinization and Ovulation

Studies using microarrays in the pig, cow, and mouse have revealed several genes in granulosa cells of the preovulatory follicles that are associated with ovulation (Jiang *et al.*, 2004; Friedmann *et al.*, 2005; Richards, 2006). Before LH surge, porcine granulosa cells have been shown to express genes related to the proliferation, active metabolism and oxidative stress responses, whereas, after LH surge, these cells expressed genes related to non-proliferation, migration and angiogenesis (Agca *et al.*, 2006). A comprehensive list of ovulation specific genes in follicular cells of cow, mouse, rat, human, monkey and pig has been provided elsewhere (Richards, 2006); however, the gene list needs to be updated.

The expression of genes in granulosa cells changes within minutes to hours after LH surge. In mouse preovulatory follicle, granulosa cells express genes within 1 h after LH surge

and known as early response genes (Carletti & Christenson, 2009). The expression of 57 early response genes up-regulated and some of these genes were related to EGF-like ligands (amphiregulin, epiregulin, and epigen), transcription factors (B-cell translocation 1 (BTG-1, and -2), early growth response-1 (EGR-1), nuclear receptor subfamily 4 group A member 1 (NR4A1 and NR4A2), hairy enhancer of split-related with YRPW motif 1 (HEY-1) and GADD45B. Also, steroidogenic (CYP19A1), angiogenic (coagulation factor F3) and inflammatory genes (PTGS2 or COX2) up-regulated after LH within few minutes (Carletti & Christenson, 2009). Analyses of the genes expressed at later time points after LH have been documented in rat and mouse by other researchers (Espey & Richards, 2002; Hennebold, 2004; McRae *et al.*, 2005).

Considering that the ovulation is controlled by genes that are temporally and transiently expressed after LH surge, microarrays were used by researchers to identify those genes in granulosa cells. In cattle, granulosa cells of preovulatory follicles were compared between three time points, i.e. 2 h before LH surge, 6 h and 22 h after LH surge to identify early (6 h) and late (22 h) response genes (Gilbert *et al.*, 2011). Early response to LH was associated with the preparation of ovulation such as response to a gonadotropin (LH), vascularization, and lipid synthesis. Late response of granulosa cells to LH was associated with luteinization and secretion such as protein localization, and intracellular transport (Gilbert *et al.*, 2011). In another study, the impact of LH surge on gene expression of granulosa cells was determined to assess markers of oocyte competence (Gilbert *et al.*, 2012). The authors concluded that the gene expression in granulosa cells before LH surge can assess bovine oocyte competence accurately compared to those after LH surge (Gilbert *et al.*, 2012). In granulosa cells of bovine preovulatory follicle, the expression of novel genes such as ADP-ribosylation factor GTPase-activating protein 3 (ARFGAP3), FST, GJA1, IDH3, INHBA, LHR, LHR lacking exon 10, polycomb responsive

complex-1 (PRC1), serglycin (SRGN), replication protein A2 (RPA2), SCD, and the TRIB2 down-regulated 23 h after hCG treatment (Ndiaye *et al.*, 2005). Most of those genes in bovine granulosa cells were related to follicular growth, ovulation and luteinization (Ndiaye *et al.*, 2005). There are certain granulosa cell genes that are classical in the expression after LH surge and down-regulate (CYP19A1, FSH-R, CYP11A1, LRP8 and SERPINE2) during the bovine periovulatory period (Ndiaye *et al.*, 2005).

In human, gonadotropin-induced genes were in granulosa cells were related to cell signaling such as adenylyl cyclase-7 and -9 (ADCY7/9), cAMP dependent phosphodiestrase (PDE) and a negative regulator of G-protein cell signaling (RGS16), cytoskeleton, and steroidogenesis (Sasson *et al.*, 2004). Biomarkers for oocyte competence and a successful pregnancy in women have been studied in granulosa cells and cumulus cells (Hamel *et al.*, 2008; Hamel *et al.*, 2010; Assidi *et al.*, 2011). The best predictors of pregnancy were UDP-glucose pyrophosphorylase-2 (UGP2) and pleckstrin homology-like domain, family A, member 1 (PHLDA1) (Hamel *et al.*, 2010). Increased expression of UGP2 indirectly regulates synthesis of glycosaminoglycans like hyaluronan by the granulosa cells in response to LH (Magee *et al.*, 2001). Hyaluronan-treated bovine embryos have high post-transfer survival rates in receipiet cows (Block *et al.*, 2009). Increased expression of the PHLDA1indicates early leutinization of human granulosa cells associated with competent oocyte to prevent apoptosis (Hamel *et al.*, 2008)

Summary

The acquisition of FSH receptors by granulosa cells allows the follicles to respond to the cyclic rise of FSH and emerge from the pool of ovarian follicles. Certain autocrine and paracrine factors such as IGF(s), EGF, TGF, FGF and estrogen synthesizing enzymes (CYP11A1 and CYP19A1) are also involved in the emergence of follicular wave. The selection of a

dominant follicle occurs when a drop in plasma FSH occurs due to the action of follicular products (inhibins and follistatin). Also the dominant follicle has acquired LHR by this time, so can continue its growth while subordinates regress. Apoptosis and atresia of the follicles result in 1) decreased intrafollicular estradiol concentrations, 2) high amounts of androgens from theca cells, 3) decrease in estradiol receptors on granulosa cells, and 4) expression of Fas antigen for FasL.

Granulosa cells of the growing dominant follicle before LH surge 1) acquire the maximum steroidogenic capacity to to process lipid and cholesterol and to produce estradiol and, 2) proliferate quickly under the influence of growth factors (e.g. IGF, and activins), gonadotropins (via the cAMP-PKA pathway), and estradiol. This results in increased DNA replication and mRNA expression of PCNA in granulosa cells. After the LH surge, the granulosa cells 1) rapidly but transiently inhibit cell cycle by inhibiting the transcription of cyclin D2 and E2 through the expression of cdK inhibitors, 2) synthesizes progesterone rather than estradiol and express high mRNA levels of STAR, HS3DB and CYP11A1, and 3) express early and late response genes that are critical for differentiation and luteinization. Finally, the process of ovulation in granulosa cells is governed by three P's i.e. progesterone, prostaglandin and proteases.

1.5 Maternal age and Fertility

1.5.1 Women

Demographics of the human population around the world and in Canada suggest that fertility decreases with increasing age of women. The risk of childlessness (proportion) in women increased steadily, i.e., 6% at the age of 20-24 years, 15% at age 30-34, 30% at age 35-39, and 64% at age of 40 to 44 years (Menken *et al.*, 1986). Also, a progressive decline in

pregnancy rate with increasing age of women after assisted reproduction has been documented (David *et al.*, 1980; David *et al.*, 1980). Increased intrauterine pregnancy losses for women at age 40 (33%) have been reported as compared to women at age < 35 (10.4%) (Gunby & Daya, 2006). Furthermore, a dramatic increase in pregnancy loss for women aged > 40 (52%) was observed compared to women at age < 30 (15%) (Stein, 1985). Similarly, the percentage of embryo transfers resulting in live child birth decreased from 50% in women at age 30 to less than 6.7% in women at age 43-44 years (ASRM, 2008).

1.5.2 Cattle

Higher fertility rate was seen in beef cows at the age of four but declined thereafter until the age of 10 years (Belling, 1963). Similar trends of decreased fertility were observed in earlier reports in dairy cattle (Tanabe & Salisbury, 1946; Herman, 1956). In zebu cattle, percentage of live births decreased from 73% at age of 2-3 years to 53% at age > 6 years (Macfarlane & Goodchild, 1973). Considering the ovarian characteristics and reproductive performance, a study demonstrated that 50% of the cows in the herd became infertile at the age of 13 years (Erickson *et al.*, 1976). In another study, fewer embryos and a higher proportion of uncleaved zygotes or unfertilized oocytes were recovered from aged cows than their daughter after ovarian superstimulation (Malhi *et al.*, 2007). In addition, follicular and endocrine profiles of aged cows were related to the peri-menopausal women (Malhi *et al.*, 2005; Malhi *et al.*, 2006; Malhi *et al.*, 2008).

1.5.3 Rodents (mouse and rat)

A decline in reproductive capacity of rat and mice has been reported due to increase maternal age (Harman & Talbert, 1970; Harman & Talbert, 1974). The number of ovulation per cycle, implantation rate and litter size reduced in mice by age ≥10 months (Jones & Krohn,

1961; Harman & Talbert, 1970; Harman & Talbert, 1974). Another study reported that fertility in three strains of rats decreased with age (Ingram *et al.*, 1958). Similarly, multiparous female rats at the age of 6 month or older showed a progressive decline in the number of live births and litter size (Matt *et al.*, 1987). The number of blastocysts also declined in multiparous female rats during 4-6 months of age (Matt *et al.*, 1987). A number of studies have highlighted age-associated changes in patterns of the estrous cycle, fecundity, ovulation, and hormone secretion of aging female rats (Lapolt *et al.*, 1986; Anzalone *et al.*, 2001; Tsai *et al.*, 2004). Unfortunately, the effects of maternal age were often studied in one general mouse strain i.e. C57BL/6 (Austad, 2003) and the research about infertility was completed in other mice strains that were engineered to study the gene mutations (Sprott, 2011).

Summary

In mammals, fertility declines with age. Assisted reproductive techniques cannot substitute the decline in fertility.

1.6 Mechanisms of Ovarian Aging:

1.6.1 Free Radical Theory

Free radical theory of aging was first proposed by Denham Harman (Harman, 1956). Reactive oxygen species (hydrogen per oxide and hypochlorite) or free radicals (superoxide, nitric oxide, and hydroxyl radical) cause oxidative damage to nucleic acid, proteins, and membranes within cells. Free radicals are produced as byproducts of oxidative metabolisms (Pryor, 1978).

Role of oxidative stress in female reproduction is highlighted in several reports (Agarwal *et al.*, 2005; Agarwal *et al.*, 2005; Agarwal *et al.*, 2012; Li *et al.*, 2012; Misa Imai *et al.*, 2012; Lord & Aitken, 2013). Oxidative damage accumulates with age and activity of free

radicals increases in human follicular fluid (Wiener-Megnazi *et al.*, 2004). Conversely, the levels of free radical scavengers such as superoxide dismutase-1 and 2 (SOD1 and SOD2), and catalase decrease in granulosa cells of aged women compared with those from young women (Tatone *et al.*, 2006). Consequently, oocytes from aged women are susceptible to oxidative damage and are indicated by increased levels of 8-hydroxydeoxyguanosine (Miyamoto *et al.*, 2010) and decreased levels of glutathione-S-transferase and thiols (Tarin *et al.*, 2004). The process of aging accelerates in fresh oocytes after exposure to the reactive oxygen agents (Miao *et al.*, 2009). In women age > 37, lower levels of total antioxidant capacity in follicular fluid are associated with unsuccessful fertilization (Carbone *et al.*, 2003). In aging mice ovary, there is an increase in oxidative damage and a concomitant reduction in anti-oxidant gene expression (Lim & Luderer, 2010); suggesting that oxidative damage is caused by an imbalance between oxidative and anti-oxidative mechanisms.

1.6.2 Mitochondrial Damage/Mutation

Mitochondria are the power house of a cell and are involved in cell proliferation and apoptosis (Li *et al.*, 2012). Age-associated decline in cellular respiration results in increased intra-mitochondrion electron leakage, high production of reactive oxygen species and reduced levels of anti-oxidant enzymes (Miquel *et al.*, 1980; Li *et al.*, 2012). In turn, the stability of mitochondrial DNA and mitochondrial functions are compromised (Li *et al.*, 2012). Age-related decay in oocytes has been shown to accompany mitochondrial dysfunction which in turn induces cellular damage (Liu & Keefe, 2000; Liu *et al.*, 2000; Ottolenghi *et al.*, 2004). In aging oocytes from mice, increased expression of genes related to mitochondrial electron transport chain and decreased mRNA levels of genes related to energy production supports the notion that aging oocytes have reduced levels of ATP than younger oocytes (Hamatani *et al.*, 2004). Likewise,

high levels of mitochondrial DNA deletion in luteinized granulosa cells of women aged > 38 years have been reported (Seifer *et al.*, 2002). Analysis of the ultrastructure of granulosa cells from aged women revealed defects in mitochondria when compared to those from young women (Tatone *et al.*, 2006). Similarly, increased number of granulosa cells with damaged mitochondria were identified in cohort of primordial follicles from aged women (de Bruin *et al.*, 2004). Age-related metabolic and morphological defects have also been reported in mitochondria of the oocyte from mouse and hamster (Hamatani *et al.*, 2004; Simsek-Duran *et al.*, 2013)

1.6.3 Advanced Glycation End Products (AGEs)

Glycation is an irreversible and non-enzymatic process of protein glycosylation that leads to the formation of AGEs (Li *et al.*, 2012). AGEs are the lipids or proteins that become glycated after exposure to sugars and cause damage to the cells by binding to the receptors termed as RAGE (receptors for advanced glycation end products), and by protein crosslinking (Schmidt *et al.*, 2000; Thomas *et al.*, 2005). RAGE has been detected in human granulosa cells, theca interna, and ovarian endothelial and stromal cells (Diamanti-Kandarakis *et al.*, 2007). AGEs-RAGE binding results in intracellular stress by activation of oxidative stress related pro-inflammatory transcription factors NF-KB (Schmidt *et al.*, 2000; Ramasamy *et al.*, 2012). Ovaries of aged mice have been shown to express decreased active detoxifying enzymes e.g. glyoxalase-1 and 2 (GLO1 and GLO2) against methylglyoxal (MG) (Tatone *et al.*, 2006), which is a major precursor of AGEs (Thornalley, 2008). Increased levels of pentosidine, a biomarker for AGEs, were observed in the primordial, primary and atretic follicles of premenopausal women (Matsumine *et al.*, 2008). In addition, MG cytotoxicity in mouse oocytes increased with age and the capacity of the cumulus cells to protect oocyte against MG toxicity decreased (Tatone *et al.*, 2011). Rate of oocyte maturation, fertilization and *in vitro* embryonic

development has been shown to reduce by MG induced toxicity or apoptosis (Tatone & Amicarelli, 2013). In addition, toxic AGEs (TAGE) levels in serum and follicular fluid have been correlated negatively with follicular growth, fertilization and embryonic development, but were positively correlated with age (Jinno *et al.*, 2011). Age-related increase in TAGE has not been reported yet in bovine follicular fluid.

1.6.4 Telomeres Length and Telomerase Activity

Telomeres is DNA sequence (TTAGGG) that repeats at the chromosomal ends to prevent end-to-end fusion of chromosomes (Blasco *et al.*, 1999). Telomere length is considered a biomarker of cellular aging because it shortens with successive cell division and results in genomic instability, cell cycle arrest and apoptosis after reaching threshold (Li *et al.*, 2012; Kalmbach *et al.*, 2013). Telomerase maintain telomere length and ensure genomic stability (Mason *et al.*, 2010). Oxidative stress and telomerase deficiency over the prolonged period may lead to a rapid shortening of telomeres and age related infertility (Keefe *et al.*, 2006). In another report, telomere length in human oocytes has been suggested as a measure of cytoplasmic fragmentation in embryos and is consistent with telomere theory of reproductive aging in women (Keefe *et al.*, 2005). Telomere function is essential for meiosis as telomere shortening via genetic manipulation reduces synapsis and recombination in mice (Liu *et al.*, 2004). Oocyte from women failing to conceive after *in vitro* fertilization had a shorter length of telomeres than those who did conceive (Keefe *et al.*, 2007). In addition, women with diminished ovarian reserves had shorter telomeres and reduced telomerase activity in granulosa cells (Butts *et al.*, 2009). These findings suggest that the telomere theory of reproductive aging can explain the age related oocyte dysfunction.

1.6.5 Ovarian Reserve

Ovarian aging is characterized by decrease in the number of primordial follicles. The ovaries of cow, sheep, pig, and women are endowed with non-replenishable pool of follicles that decline with age (Evans *et al.*, 2012). Oocyte quality declines with age and causes a higher incidence of aneuploidy (te Velde *et al.*, 1998; te Velde & Pearson, 2002). Decline in ovarian follicles also resulted in infertility, irregular menstrual cycle and menopause in women (de Bruin *et al.*, 2004). Women with decreased ovarian reserve had compromised follicular environment, follicular fluid metabolites, cell metabolism and progesterone production (Pacella *et al.*, 2012). Women approaching the age of menopause (average 51 years) have fewer than 1000 follicles in the ovaries (te Velde & Pearson, 2002). In cattle, size of ovarian reserves has been correlated with fertility and ovarian functions via alteration in intrafollicle estradiol production (Ireland *et al.*, 2009). Assessment of ovarian reserve can be done by counting the number of antral follicles > 3 mm in bovine ovary using ultrasonography (Ireland *et al.*, 2008). In aged cows (13-14 years) compared to their daughters (1-4 years), number of 4-5 mm follicles at the time of wave emergence were lower, however number of 2-3 mm and 6-8 mm follicles did not differ between the age groups (Malhi *et al.*, 2005). Infertility treatment relies on the assessment of ovarian reserve and chronological age is the single most important indicator (Jirge, 2011). However, other endocrine parameters (FSH, inhibins, AMH and estradiol) and antral follicle counts are also used and have been reviewed elsewhere (Ramalho de Carvalho *et al.*, 2012).

Summary

Mechanisms that are involved in ovarian aging include 1) free radicals mediated oxidative damage, 2) mitochondrial dysfunction that causes an increase in mRNA levels of genes related to the electron transport chain and decrease in mRNA of those related to energy

production, 3) advanced glycation end products that increase oxidative stress and the expression of proinflammatory transcription factor NFKB, 4) shortening of telomere due to the prolonged deficiency of telomerase, and 5) diminished ovarian reserve that results in a higher incidence of aneuploidy and infertility.

1.7 Bovine Model of Maternal Reproductive Aging

1.7.1 Follicular and Endocrine Dynamics in Reproductive Women

In women, menstrual cycle is composed of approximately 28 days with 14-21 days of follicular phase and 14 days of luteal phase. The follicular phase is marked by onset of menses and ends the day before LH surge. The luteal phase begins from the day of LH surge and ends on the first day of menses. According to the most recent follicular wave theory in women, a total of 4-14 antral follicles of 4-5 mm in diameter emerge 2 or more times during the cycle in wave like pattern (Baerwald *et al.*, 2003). About 60% of women have been reported having 2-wave while 31% of the women have 3-wave follicle patterns during the menstrual cycle (Baerwald *et al.*, 2003). Recruitment of the follicles in a wave occurs with the transient rise in FSH during early follicular or late luteal phase (Baerwald *et al.*, 2003). Follicle selection occurs once in early-to mid-follicular phase (Zeleznik, 2004). The divergence of the dominant follicle (~10 mm in diameter) occurs around day 6-9 of the follicular phase (Baerwald *et al.*, 2003). Anovualtory waves emerge during early and mid-luteal phase while the ovulatory wave emerges during early follicular phase (Baerwald *et al.*, 2003). Antral follicles during the luteal phase are atretic due to influence of corpus luteum (McNatty *et al.*, 1983).

Life span of corpus luteum (CL), progesterone secretions and estradiol concentrations during the luteal phase were not different in women with two or three waves (Baerwald *et al.*, 2005). Preovulatory follicle grows at the rate of 1-4 mm/day and reaches 16-29 mm in diameter

before ovulation (Bakos *et al.*, 1994; Baerwald *et al.*, 2009). About 90% of the estrogen is produced by the dominant follicle during preovulatory period (Baird & Fraser, 1974). Aromatase and IGF-2 mRNA levels increase in the preovulatory follicle and are inversely related to intrafollicular concentration ofAMH (Geisert *et al.*, 1991; Nielsen *et al.*, 2010).

Estradiol concentrations increase during luteal and early follicular phase of the cycle despite anovulatory follicle wave (Baerwald *et al.*, 2003). Estrogen levels peaks the day before LH surge (Bomsel-Helmreich *et al.*, 1979). Ovulation occurs within 24 h after LH surge (Kerin *et al.*, 1981). Serum progesterone concentrations rise after preovulatory estradiol peak but before LH surge (Chikazawa *et al.*, 1986; Westergaard *et al.*, 1986). Inhibin B secretions from granulosa cells peak during mid-follicular phase and 2-days after mid cycle LH surge (Groome *et al.*, 1996). It has been suggested that the CL produces estradiol and Inhibin A during mid-luteal phase of the menstrual cycle (Groome *et al.*, 1996).

1.7.2 Follicular and Endocrine Dynamics in Peri- and Post-menopausal Women

Changes in follicular and endocrine dynamics during the peri- and post- menopause have been characterized by Staging of Reproductive Aging Workshop; STRAW (Harlow *et al.*, 2012). Peri-menopusal stage spans from early-and late-menopause transition to early post-menopause stages (Harlow *et al.*, 2012). In early-menopause transition, menstrual cycles are variable in length with the persistent difference of 7 days or more between consecutive cycles (Harlow *et al.*, 2012). Circulating concentrations of FSH are elevated during early follicular phase, but concentrations of AMH, inhibin B and number of antral follicle are low (Harlow *et al.*, 2012). In late menopausal transition, amenorrhea of 60 days or longer occurs and is accompanied by increased cycle variability and prevalence of anovulation (Harlow *et al.*, 2012). Antral follicle count, inhibin B and AMH concentrations are low (Harlow *et al.*, 2012). Plasma

FSH drastically changes from the levels that are characteristic of early reproductive years to menopausal range (Harlow *et al.*, 2012). However, FSH concentration greater than 25 IU/L in random blood sample are the characteristic of this stage (Stricker *et al.*, 2006). These changes last for 1-3 years and may include vasomotor symptoms such as hot flashes; night sweats, mood and sleep disturbances (Harlow *et al.*, 2012). Erratic changes in estradiol concentrations and decreased luteal progesterone is reported in aged women (Santoro *et al.*, 1996; Hale & Burger, 2009) .

Early post-menopause is further subdivided into three sub-categories (A, B and C) (Harlow *et al.*, 2012). In early post-menopause categories A and B, FSH levels continue to increase while estradiol concentrations continue to decrease until 2 years after last menstrual period (Randolph *et al.*, 2011). Antral follicle count is very low along with low levels of inhibin B and AMH (Harlow *et al.*, 2012). These symptoms last for one year and most likely accompany vasomotor symptoms (Randolph *et al.*, 2011). Category C persists for 3-6 years after the final menstrual period during which estradiol and FSH stabilize (Harlow *et al.*, 2012). Conversely, concentrations of inhibin B and AMH are very low, and antral follicle count is also very low (Harlow *et al.*, 2012). Late post-menopause stage manifest limited changes in endocrine functions and accompany symptoms such as vaginal dryness and urogenital atrophy (Dennerstein *et al.*, 2000). At this stage, plasma FSH concentrations decrease in aged women (Hall, 2004).

1.7.3 Superovulatory Response in Aged Women

Controlled ovarian stimulation with clomiphene citrate or gonadotropin along with intrauterine insemination is the first choice to treat subfertility in aged women (Harris *et al.*, 2010). Very limited information is available regarding the outcome of the ovarian stimulation protocols in women aged > 40 years. Data from 168 patients (aged > 40 years) undergoing a total

of 469 cycles of treatment with clomiphene citrate or gonadotropin followed by intrauterine insemination revealed that out of 402 completed cycles, only 28 clinical pregnancies were reported (Corsan *et al.*, 1996). Fecundity rates in women aged 40, 41 and 43 were 9.6, 5.2 and 2.4%, respectively (Corsan *et al.*, 1996). Considering the live birth rates, *in vitro* fertilization (13.7%) has been proposed as a better alternative to gonadotropin induced ovarian stimulation (2.6%) in women at 40 years of age (Wiser *et al.*, 2012).

In vitro fertilization (IVF) induce considerable attrition in oocyte, therefore, controlled ovarian stimulation increase the chances of producing good quality embryos (Lekamge *et al.*, 2008). Chances of live birth decrease if less than 5 oocytes are processed for IVF (Sharma *et al.*, 2002). Standard approach for the prediction of ovarian stimulation response depends on the age and early follicular phase FSH levels (Lekamge *et al.*, 2008). In women with normal plasma FSH levels and aged < 36 years, ovaries are stimulated with 150 IU/day of FSH whereas the women with diminished ovarian reserve and age > 36 years are stimulated with 200-300 IU/day (Popovic-Todorovic *et al.*, 2003). However, others have argued that the starting dose should be customized according to the patients based on the age, antral follicle count, plasma AMH, FSH and inhibin B levels (de Vet *et al.*, 2002; Muttukrishna *et al.*, 2005; Smeenk *et al.*, 2007).

1.7.4 Oocyte Competence in Aged Women

Meiotic abnormalities due to maternal age include inability of oocyte to resume meiotic maturation and/or errors in meiotic maturation (Armstrong, 2001). Human oocytes are prone to meiotic errors and donor age increases the frequency of errors in chromosome segregation in in-vitro matured oocytes (Volarcik *et al.*, 1998). Age-related changes in gene expression of mature human oocytes have been associated with cell cycle regulation, chromosomal alignment, sister chromatid separation, oxidative stress and ubiquitination (Grondahl *et al.*, 2010). Other age-

associated reasons that influence the competence of human oocyte include the changes in mitochondrial structure and function, cell cycle deregulations, oxidative stress and epigenetics (Tatone, 2008).

1.7.5 Follicular and Endocrine Dynamics in Aged Cattle

Follicular and endocrine characteristics of aged cattle have been documented (Bryner *et al.*, 1990; Malhi *et al.*, 2005). In aged beef cows (13-14 years), fewer numbers of 4-5 mm follicles were recruited into wave as compared to their daughters (1-4 years), however, no difference in 2-3 mm follicle per wave was detected between the age groups (Malhi *et al.*, 2005). The diameter of the ovulatory follicle was smaller in 2-wave cycle in aged cows than their daughters whereas no age related effect was found in 3-wave cycle (Malhi *et al.*, 2005). The day of wave emergence, interovulatory interval, and interwave interval did not differ between aged cows and daughters. Likewise, the CL diameter from Day 0 through Day 15 tended to be smaller in 2-wave cycle in aged cows than their daughter whereas no such age related effect was detected in 3-wave cycle (Malhi *et al.*, 2005).

In aged cows, plasma FSH concentrations were higher during the interovulatory interval than their daughters (Malhi *et al.*, 2005). Mean plasma LH concentrations did not differ between the age groups during the interovulatory interval or during preovulatory surge (Malhi *et al.*, 2005). Luteal phase progesterone concentrations tended to be lower in aged cows than their daughters (Malhi *et al.*, 2005). Estradiol concentrations 7-days preceding ovulation were higher in aged cows than their daughters, but the preovulatory peak estradiol concentrations did not differ between the age groups (Malhi *et al.*, 2005). In another study, there was no difference in the duration of the estrous cycle between young (5-7 years) and old cows (12-13 years) (Bryner *et al.*, 1990). Endocrine patterns of progesterone and FSH during the estrous cycle between aged

and young lactating beef cows partially disagreed from the conclusion of Malhi *et. al.* (2005) i.e. progesterone levels were lower in aged cows on Day 14 and Day 15 of estrous cycle and FSH levels rose early in aged cows from the day of estrus until the day 6 of the cycle compared to those from young cows. However, the patterns of estradiol and LH were similar in both studies (Bryner *et al.*, 1990; Malhi *et al.*, 2005). Moreover, endocrine changes were not temporally associated with follicular waves dynamics in earlier study (Bryner *et al.*, 1990) and may be the cause of disagreement between the two studies.

1.7.6 Superovulatory Response in Aged Cattle

Superovulatory response has been evaluated in aged cows (Malhi *et al.*, 2006; Malhi *et al.*, 2008) and indicates that aged cows have the tendency of fewer ovulatory follicles after ovarian stimulation with gonadotropin (Malhi *et al.*, 2006). Furthermore, LH surge has been reported being delayed in aged cows (13-16 years) than their daughters (3-6 years) (Malhi *et al.*, 2006). In aged cows, ovarian stimulation with gonadotropin resulted in fewer > 6 mm follicles as well as less number of ovulation compared to their daughters (Malhi *et al.*, 2008). Likewise, the mean number of oocytes and embryos were affected by the age of the donor beef cows (Breuel *et al.*, 1991). The number of non-ovulatory follicles has been shown to increase from five to nine years of age in Slovak Pinzgau cattle breed after ovarian stimulation with pregnant mare serum gonadotropin (PMSG) (Kacmarik *et al.*, 1987). Earlier, donor age from two years to 14 years has been proposed to have a little impact on the ovarian response to gonadotropin; however, cut off of nine years have been recommended beyond which ovarian response is expected to decline (Donaldson, 1984; Kafi & McGowan, 1997). The conclusions from these studies suggest that the age of the cow influences the ovarian response to gonadotropins.

1.7.7 Oocyte Competence in Aged Cattle

Oocyte competence is defined by the capability to resume meiosis, to be fertilized and cleaved, to develop into blastocysts and establish a pregnancy (Armstrong, 2001). In comparison to the young cows, fewer numbers of embryos were recovered from aged cows despite no difference in ovulation and corpora lutea (Malhi *et al.*, 2007). Higher percentage of unfertilized oocytes or uncleaved zygotes were recovered from aged cows (71%) than their daughters (31%) (Malhi *et al.*, 2007). Similarly, aged-associated decreases in cleavage and blastocyst rates were observed in oocytes of the crossbred (Murray Grey x Brahman) aged cows (15 years) compared with young cows (12 months old) after retrieval through ovum-pickup (Su *et al.*, 2012). Oocytes at germinal vesicle (GV) or meiosis-II stage (M-II) from aged vs. young cows revealed differences in gene expression related to oxidative phosphorylation, mitochondrial and the protein dysfunctions (Takeo *et al.*, 2013).

In comparison to the young cows, oocytes from aged cows showed fast progression of nuclear maturation, activation of maturation promoting factor (MPF) and abnormal rate of fertilization (Yamamoto *et al.*, 2010). Authors suggested that premature maturation in oocytes from aged cows may be due to impaired oocyte and cumulus cell gap junctions (Yamamoto *et al.*, 2010). The morphological parameters of the oocytes do not change considerably with age despite the decrease in developmental potential of the oocytes (Katska & Smorag, 1984). Recently, analysis of mature oocytes from aged cows indicates the decreased mitochondrial DNA copy number as compared to those from young cows (Iwata *et al.*, 2011). Maternal age was positively correlated with ATP contents of mature oocytes as well as with abnormal fertilization rate in the bovine oocytes (Iwata *et al.*, 2011). In contrast, results of another study indicate 30% less cytoplasmic ATP contents in vivo mature oocytes of aged cows compared to

the young cows (Dadarwal, 2012). All of these findings suggest that developmental competence of the oocyte is compromised due to advance reproductive age of the cattle.

Summary

Follicular and endocrine dynamic in healthy reproductive women resemble with normal cycling cows. Considering these similarities and convenience of obtaining tissues for testing research hypotheses, bovine model of reproductive aging was proposed to understand the follicular and luteal dynamics in aged women (Malhi *et al.*, 2005). The follicular and endocrine dynamics, synchronization and superovulatory response and oocyte competence in bovine model resembled peri-menopausal women. Hence, this model provides an excellent opportunity to understand the molecular basis of reproductive aging.

1.8 Comparison of Modern mRNA Quantification Techniques

Global and limited gene expression profiling of oocytes and ovarian somatic cells allow insight about follicular development and aging. Techniques that have been employed to measure the changes in mRNA of the transcripts are summarized in Table 1.2.

In this thesis, global analysis of gene expression of granulosa cells from aged and young cows was done using bovine specific microarrays. In addition, differences in gene expression of granulosa cells between aged and young cows were confirmed by RT-qPCR. Microarrys were used because it allowed to determine the changes in gene expression of several thousands genes simulateously at a given point in time. As a result, molecular and cellular functions and pathways were identified in granulosa cells of the dominant follicles that were affected by the increase in maternal age.

Table 1.2 Comparison of various mRNA quantification techniques.

	Pros	Cons
RT-PCR	• Highly sensitive • Amplification of mRNA requires few bases • Highly effective for absolute and relative mRNA quantification	• Quantification depends on invariant control or exogenous synthetic control calibration (optimization) • Require good RNA integrity (non-degraded) and quality • Unable to detect transcript variants and transcript size
Northern Blotting	• Low-tech equipment and method • Less manipulation of RNA (enzymatic degradation) • Multi-level sample progress evaluation • Ideal for detection of splice variants and transcript size • Allows usage of different kinds of probes	• Extremely sensitive to RNA degradation • Least sensitive • Difficult to handle with multi-probes
In situ hybridization	• Localize mRNA expression within the cell • Tolerate partially degraded RNA	• Unable to detect splice variants and transcript size • Require high degree of optimization
Nuclease protection assay	• Sensitive than northern blotting • Tolerate partially degraded RNA • Can detect members of multiple gene families	• Unable to detect splice variants and transcript size
Microarray	• Profiles global gene expression • Based on the hybridization of specimen target strands onto the immobilized complementary probe strand.	• Bias due to amplification and variable due to hybridization and labeling efficiencies • High background noise may affect quantification
Next generation sequencing	• Accurately detect transcripts number based on deep sequencing • Quantification is more robust for global gene expression • Allows detection of alternate splice variants • Very low background signals • Independent of prerequisite knowledge of reference transcriptome • High dynamic range of expression levels	• Overwhelming ribosomal RNA • Short reads • Less base accuracy • Variation in read density along the length of the transcripts

Modified from (Kogenaru *et al.*, 2012) and Life Technologies https://www.lifetechnologies.com

2 CHAPTER 2: OBJECTIVES AND HYPOTHESIS

Overall objective of the study was to determine the maternal age-associated changes in transcriptome of granulosa cells of bovine dominant follicles. Earlier studies in cattle documented that the time of follicular wave emergence, diameter of dominant follicles at the time of follicle selection (Day 3 after ovulation) and the interovulatory interval did not differ between old cows and their daughters (Malhi *et al.*, 2005). On the other hand, diameter of preovulatory follicle was smaller in old cows compared to their daughters (Malhi *et al.*, 2005). In addition, LH surge was delayed in aged cows (Malhi *et al.*, 2006) and higher percentage (13%) of late ovulation occurred between 48-72 h compared to their daughters (4%) (Malhi *et al.*, 2008). Based on these findings, proposed overall hypothesis of the thesis is that the transcriptome difference between granulosa cells of aged and young cows will be more apparent near the time of ovulation than at the time of selection of the dominant follicle.

2.1 Specific Objectives

1. To select the stable reference gene for the relative gene expression study of bovine granulosa cells from dominant follicles and FSH-stimulated follicles.
2. To determine the maternal age-associated changes in transcriptome of bovine granulosa cells of dominant follicle at the time of selection.
3. To determine the maternal age-associated changes in transcriptome of bovine granulosa cells of preovulatory follicle 24 h after LH treatment.
4. To determine the changes in gene expression of granulosa cells between the time of dominant follicle selection and 24 h after LH in young vs. aged cattle.

3 CHAPTER 3: SELECTION OF STABLE REFERENCE GENE FOR GENE EXPRESSION ANALYSES OF BOVINE GRANULOSA CELLS

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Roles and Contributions of Co-authors: Fernanda Dias helped in tissue collection, sample processing and setting up RT-qPCR. Isabelle Dufort helped in sample processing, setting up RT-qPCR and critically reviewed the manuscript. Vikram Misra helped in setting up PCR and critically reviewed the manuscript; Marc-Andre critically reviewed the manuscript and provided reagents, and Jaswant Singh supervised, helped in study design, data analysis and critically reviewed the manuscript.

Relationship of this study to the dissertation

Using quantitative real-time PCR (RT-qPCR), relative gene expression analysis of mRNA levels of gene of interest requires normalization with the mRNA levels of another gene, known as "reference gene", in the same sample for precise measurement of gene expression. However, mRNA levels of the reference genes may vary in sample due to variety of physiological conditions. This study evaluated the mRNA levels of the two conventional and four novel reference genes in granulosa cells of the dominant follicles during maternal and follicular aging. Based on the results of this study, subsequent studies in this dissertation utilized the set of reference genes to normalize the expressions of the genes of interests in the samples for the purpose of validation of microarrays.

3.1 Introduction

Granulosa cells nourish the oocyte during its development (Brower & Schultz, 1982; Barrett & Albertini, 2010), and regulate the systemic hormonal milieu and local ovarian microenvironment by producing steroids (primarily estrogen) along with numerous autocrine and paracrine growth factors. Therefore, granulosa cells are considered an excellent candidate to identify the molecular markers of oocyte quality in cattle (Bettegowda *et al.*, 2008; Ito *et al.*, 2008), humans (Hamel *et al.*, 2008; Hamel *et al.*, 2010) and rodents (Malcuit *et al.*, 2009).

Changes in gene transcripts of bovine granulosa cells have been studied at the time of follicle selection (Evans & Fortune, 1997; Fayad *et al.*, 2004), preovulatory phase (Gilbert *et al.*, 2011) and after ovarian super-stimulation (Gilbert *et al.*, 2012) to understand molecular mechanisms involved in follicular growth and atresia, ovulation, oocyte competence and embryo development. In these and similar studies, tools such as northern blotting assay (Fayad *et al.*, 2004), suppression subtractive hybridization technique (SSH) (Fayad *et al.*, 2004), in situ hybridization (Evans & Fortune, 1997), and microarrays (Evans *et al.*, 2004; Lingenfelter *et al.*, 2008) have often been employed to quantify the levels of messenger ribonucleic acid (mRNA). In comparison to SSH and microarrays, quantitative real-time polymerase reaction (RT-qPCR) provides quantitative measurement of gene transcript levels due to higher sensitivity, reproducibility, and greater dynamic range (Ginzinger, 2002), and is often used as a "gold standard" to validate the data from these tools (Provenzano & Mocellin, 2007).

Relative quantification of mRNA of a gene of interest using RT-qPCR can be challenging due to errors such as inefficient reverse transcription (Stahlberg *et al.*, 2004), unequal amounts of starting material (mRNA or cDNA), and poor RNA integrity/quality (Fleige & Pfaffl, 2006). To control these non-biological (i.e. technical) variations, often the expression

of another gene known as a "reference" gene is quantified in the same sample, and mRNA amount of the gene of interest is adjusted or "normalized" to this reference gene. A suitable reference gene should be adequately expressed in tissues and the level of its transcripts should have minimum variability regardless of experimental conditions and tissues type (Hruz *et al.*, 2011). Recent data suggest that there is no single universally stable reference gene (Hruz *et al.*, 2011) and expression of reference genes may be altered due to the tissue type and experimental treatments (Hruz *et al.*, 2011) such as age (Chen *et al.*, 2006; Touchberry *et al.*, 2006). Therefore, geometric mean of multiple reference genes is proposed to determine the relative expression of a given gene of interest (Vandesompele *et al.*, 2002).

In cattle, reference genes have been identified for skeletal muscle (Perez *et al.*, 2008), mammary gland (Bionaz & Loor, 2007; Kadegowda *et al.*, 2009; Bougarn *et al.*, 2011), corpus luteum (Pfaffl *et al.*, 2004), endometrial tissue (Walker *et al.*, 2009), adipose tissue (Hosseini *et al.*, 2010), peripheral lymphocytes (Spalenza *et al.*, 2011), liver, kidney, pituitary and thyroid (Lisowski *et al.*, 2008; Lecchi *et al.*, 2012). However, little is known about the stable reference genes to study the relative gene expression changes in granulosa cells from ovarian dominant follicles. Usually a single and occasionally multiple reference genes have been used with or without prior validation. These reference genes include Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Machado *et al.*, 2009), Beta-actin (ACTB) (Zielak *et al.*, 2008), Acidic ribosomal phosphoprotein (RPLO) (Luo & Wiltbank, 2006) and 18s rRNA (Voge *et al.*, 2004). To the best of our knowledge, the stability of reference genes in bovine granulosa cells has not been carefully evaluated. Therefore, the objective of this study was to determine stable reference genes for relative gene expression analyses of granulosa cells from dominant and FSH-

stimulated follicles in beef cattle obtained during a wide variety of maternal and follicular aging conditions.

In this study, granulosa cells were collected 1) at two different stages of dominant follicle development (growing and preovulatory phases) from aged and young cows and 2) after three different type of super-stimulatory treatments (4d-FSH, 7d-FSH and FSH-starvation) were analyzed to determine the stable reference genes using RT-qPCR. The transcript levels of six candidate genes including two traditional reference genes (GAPDH and ACTB) and four novel housekeeping genes (EIF2B2, UBE2D2, SF3A1, RNF20) were compared for stability using GeNorm, Delta Ct, and NormFinder programs and comprehensive ranking orders of the genes was computed for comparisons within/between dominant and FSH-stimulated follicles. Finally, optimal number of reference genes was determined by GeNorm program and comparisons of Pearson's coefficient of correlations between normalization factors.

3.2 Materials and Methods

3.2.1 Collection of Granulosa cells

Experimental protocols were conducted according to the guidelines of the Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee. For maternal aging analyses, aged (17 ± 5 years; n=6) and young (8 ± 3 years; n=5) Hereford cows were synchronized for ovulation by giving single luteolytic dose of prostaglandin ($PGF_{2\alpha}$; 25 mg; i.m., Lutalyse®, Pfizer Canada Inc., Kirkland, QC, Canada). Ovulations, taken as a day of emergence of first follicular wave (Day 0), and subsequent follicular dynamics were monitored by transrectal B-mode ultrasonography using 7.5 MHz linear-array transducer (SSD 900; Aloka Co. Ltd., Tokoyo, Japan) as described earlier (Singh *et al.*, 1997; Malhi *et al.*, 2005). Granulosa cells were collected either by transvaginal ultrasound-guided follicle aspirations or

after ovariectomies (Drost *et al.*, 1992; Berfelt *et al.*, 1994). Growing dominant follicles (n=2 young cows and n=2 aged cows) at the time of selection (Day 3 after wave emergence) were used to collect granulosa cells. For obtaining granulosa cells from preovulatory follicles (n=3 young cows and n=4 aged cows), two doses of prostaglandin (Lutalyse®, 25 mg, i.m.) were given 12 h apart on Day 4.5 and 5, respectively and LH (Lutropin® V; 25 mg, i.m., Bioniche Animal Health, Belleville ON, Canada) was given on Day 6; granulosa cells were collected 24 h later (Day 7).

For FSH-stimulation and follicular aging analyses, granulosa cells were collected by employing three regimens of FSH (Folltropin® V; Bioniche Animal Health, Belleville ON, Canada) for variable duration to induce ovarian super-stimulation (Dias *et al.*, 2012). Initially, cows (n=9) were synchronized for ovulations by two doses of prostaglandin ($PGF_{2\alpha}$ analog cloprostenol, 500 µg, i.m., Estrumate, Schering-Plough Animal Health, Kirkland, QC, Canada), 12 days apart. Emergence of the follicular wave was synchronized by ablation of follicles ≥ 5 mm, five to eight days after ovulation using transvaginal ultrasound-guided follicular aspiration (Berfelt *et al.*, 1994). All cows were given an intravaginal progesterone-releasing device (CIDR-B; Pfizer Canada Inc., Pointe Claire, QC, Canada) and allocated randomly to one of the three super-stimulatory treatment groups: 1) standard FSH duration; n=3, 2) FSH starvation; n=3, or 3) long FSH duration; n=2). Cows in the standard FSH duration and FSH starvation-groups were given eight doses of FSH (Folltropin-V, 25 mg, i.m., Bioniche Animal Health, Belleville, ON, Canada) at 12 h intervals over 4 days, whereas cows in the long duration group were given 14 doses of FSH over 7 days (each dose equivalent to 25 mg of NIH-FSH-P1). Cows in the standard FSH duration group were given two doses of prostaglandin (Lutalyse®, 25 mg; i.m.) 12 h apart on third day of FSH treatment, whereas the FSH starvation and long FSH duration groups were

given prostaglandin on sixth day of FSH treatment. For all cows, CIDR devices were removed at the time of second dose of prostaglandin. All Cows were given (Lutropin® V, 25 mg, i.m.) 24 h after CIDR removal. Granulosa cells from FSH-stimulated follicles were collected after performing ovariectomies (Drost *et al.*, 1992).

3.2.2 Granulosa cells RNA extraction, amplification and cDNA synthesis

Cumulus oocyte complexes were removed from the aspirated follicular fluid and remaining aspirate was centrifuged in 1.5 ml RNase-free microcentrifuge tubes at 700 g for 5 minutes. Follicular fluid was removed and the granulosa cell pellet was either snap-frozen in liquid nitrogen or suspended in 300-700 µl of RNA stabilizing and protecting solution (RNA*later*, catalog #. AM7020; Life Technologies, Carlsbad, CA, USA) for 12 h at 4 °C. All granulosa cells were stored at -80 °C until further processing.

The extraction of total RNA of granulosa cells was performed using the RNA Isolation Kit (#. KIT0204; Life Technologies, Burlington, ON, Canada). Integrity index and concentration of total RNA were determined by 2100-Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and samples with mRNA integrity ≥ 5 were used for RT-qPCR. Linear amplification (Van Gelder *et al.*, 1990) of RNA of granulosa cells from a subset of samples (n=3; dominant follicles and n=8; FSH-stimulated follicles) was carried out using Ribo Arcturus®Amp® HS^{plus} (Kit #. KIT0525, Life Technologies, Burlington, ON, Canada) as per manufacturer's instructions. For rest of the samples (non-amplified; n=8), a total of 50 ng/µl of mRNA was reverse transcribed into cDNA by cDNA synthesis kit (Kit #. 95047-100, qscript™; Quanta Biosciences Inc. Gaithersburg, MD, USA) using the oligo-dt method as per manufacturer's instructions.

3.2.3 Selection of reference genes and primer design

Six candidate reference genes were used in this study including two common reference genes i.e. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB) and four novel reference genes i.e. Ubiquitin-conjugating enzyme-E2D2 (UBE2D2), Eukaryotic translation initiation factor-2B2 (EIF2B2), Splicing factor-3a, subunit-1 (SF3A1) and Ring finger protein-20 (RNF20). Commonly used reference genes were selected based on previous studies (Zielak et al., 2008; Machado et al., 2009) whereas novel reference genes were selected based on preliminary analysis of a microarray dataset of dominant follicles (dataset obtained from a parallel study, approximately 20000 genes; Agilent bovine chip customized for EmbryoGene Network, Canada (Robert et al., 2011). The selection criteria were: 1) high-level of expression (minimum expression value of 7 above background), and 2) expression stability (lowest coefficient of variation) among the probed known ~20,000 genes in the microarray data (Table 3.1).

Forward and reverse primers for the candidate reference genes (Table 3.2) were designed using Primer3 web interface (http://frodo.wi.mit.edu/primer3) and were analyzed for hairpins, secondary structures and compatibility using integrated DNA technologies web interface (http://scitools.idtdna.com/scitools/Applications/OligoAnalyzer/). *In silico*, specificity of primers was confirmed through BLAST against NCBI database (www.blast.ncbi.nlm.nih.gov). Primers were manufactured by a commercial company (Integrated DNA Technologies; www.scitools.idtdna.com). Efficiency and specificity of primers of each candidate reference gene were tested by analyzing dissociation curves from MxPro program (Agilent Technologies); please see next section for RT-qPCR details. Amplicon size for each gene was confirmed through 1% agarose gel electrophorsis and purified by QIAquick gel

extraction kit (Qiagen) for sequence analysis using ABI 3730 XL DNA analyzer (Applied Biosystems). Sequences from both primers were analyzed using Gap 4.4 program (Staden-package; http://www.mrc-lmb.cam.ac.uk/pubseq/manual/gap4_unix_79.html) and amplicon sequence of each gene was confirmed for specificity through BLAST against NCBI database.

3.2.4 Real-time quantitative polymerase chain reaction (RT-qPCR)

The PCR reactions were set up using Mx3005P machine (Agilent Technologies). A total of 2µl of cDNA from each sample was added to 12.5 µl of SYBR green master mix II, 1875 nM of each reverse and forward primer, 0.375 µl of reference dye and 6 µl of nuclease-free water to make a final volume of 25 µl. Three-step PCR amplification program was used i.e. initial denaturation at 95 °C for 10 minutes and for 30 seconds in following 40 cycles, a fixed annealing temperature 55 °C for 1 minute for all primers, and extension at 72 °C for 1 minute were used throughout 40 cycles. Separate PCR was set up for each gene which included its standard curve (dilutions: 10^{-2} to 10^{-9} ng/µl), non-template controls (NTC) and all granulosa cell samples in the same run (sample maximization method). Standard curve and NTC samples were run in triplicate reactions and granulosa cell samples were run as single reaction.

3.2.5 Messenger RNA levels of reference genes

Amplification efficiency for each candidate reference gene was calculated based on the slope of standard curve using MxPro program (Mx3005P®, Agilent Technologies). Cycle threshold values (CT) and relative quantities for all granulosa cell and standard curve samples were exported to Microsoft® Excel for further analysis. First, comparison of the overall mRNA levels i.e. (mean CT ± Standard deviation) of each candidate reference gene (data combined among follicle type, age, super-stimulatory treatment protocol and sample processing technique) was made using ANOVA. Next, the mRNA levels of each candidate reference gene were

analyzed according to the follicle type (dominant vs. preovulatory), age (young vs. aged), super-stimulatory protocol (short vs. long vs. starvation) and sample processing technique (non-amplified vs. amplified). Data were statistically analyzed by factorial design using the Statistical Analysis System program package (SAS version 9.2, 2002-2008; SAS Institute Inc., Cary, NC, USA).

3.2.6 Stability of reference genes

A stability of candidate reference genes in dominant and FSH-stimulated follicles was determined by GeNorm (Vandesompele *et al.*, 2002), Comparative delta CT (Silver *et al.*, 2006), and NormFinder (Andersen *et al.*, 2004) programs. These programs measured the stability value of each reference gene separately and ranked them from most stable to least stable. Briefly, Comparative Delta CT program (Silver *et al.*, 2006) compared relative expression of "pairs of genes" using CT values and calculated "average standard deviation" as a measure of stability (i.e., the gene with lowest average standard deviation value was ranked as most stable). NormFinder program (Andersen *et al.*, 2004) identified the stable reference genes using a statistical linear mixed modelling based on relative quantities of amplicon in the samples. GeNorm^{plus} program (Vandesompele *et al.*, 2002) (www.biogazella.com) was used to calculate stability measure (M-value) for each reference gene based on pairwise comparison model (Vandesompele *et al.*, 2002). Reference candidate gene with minimum M-values was considered as most stable reference gene.

Initially, the ranking orders of candidate reference genes were analyzed for dominant follicles and FSH-stimulated follicles separately and then by combining the two follicle categories to determine the most stable genes.

3.2.7 Comprehensive ranking order of reference genes

Comprehensive ranking orders of the reference genes for granulosa cells of dominant and FSH-stimulated follicles (separate and combined analyses) were determined with RefFinder program(http://www.leonxie.com/referencegene.php). Comprehensive ranking order is based on the geometric mean of assigned weights according to the ranking order of an individual gene by GeNorm, delta CT and NormFinder programs. Reference gene with least geometric mean of assigned weights was ranked as most stable.

3.2.8 Optimal number of reference genes

GeNorm program was used to determine the optimal number of reference genes that are required to calculate the normalization factor (NF) for granulosa cells of dominant and FSH-stimulated follicles (separate and combined analyses). Briefly, pairwise variation (V_n/V_{n+1}) between two sequential normalization factors (NF_n/NF_{n+1}) was calculated until the variation V_n/V_{n+1} dropped below a threshold of < 0.15. To determine if a unified set of genes could be used for analysis of dominant and FSH-stimulated follicles, normalization factors were recalculated (NF recalculated) for the dominant and FSH-stimulated follicles datasets based on the gene order recommended from the combined analysis (dominant + FSH-stimulated follicles). Then Pearson's correlations between NF recalculated vs. NF minimum for the granulosa cell samples of dominant or FSH-stimulated follicles compared the two sets of normalization factors.

Table 3.1 Microarray data showing mean expression levels (signal intensity; A value) and coefficient of variation (CV) for candidate reference genes. Expression levels in granulosa cells of growing dominant follicles and preovulatory follicles in aged vs. young.

Bovine embryo gene feature ID	Gene Symbol		Growing dominant follicle		Preovulatory dominant follicle		Combined dataset
			Aged (n=3)	Young (n=3)	Aged (n=3)	Young (n=3)	(n=12)
EMBV3_38516	EIF2B2	Mean	9.1	8.9	8.8	8.5	8.8
		CV	0.03	0.01	0.04	0.04	0.03
EMBV3_00800	RNF20	Mean	10.8	10.8	10.6	10.9	10.8
		CV	0.07	0.03	0.03	0.07	0.04
EMBV3_33523	SF3A1	Mean	11.6	11.7	11.5	11.3	11.5
		CV	0.03	0.03	0.01	0.08	0.04
EMBV3_13319	UBE2D2	Mean	13.2	13.4	13.5	13.3	13.4
		CV	0.01	0.02	0.01	0.03	0.02

Table 3.2 List of candidate reference genes and the details about primer pair sequences, amplicon size and PCR efficiency of each gene that were used in the study.

Gene	Function	Accession Number	Oligo	Primer pair Sequence (5' to 3')	Amplicon size (bp)	Efficiency (%)
ACTB	Cytoskeleton	NM_173979.3	Reverse	TTGAAGGTAGTTTCGTGAATGC	211	93.4
			Forward	GTGACATCAAGGAGAAGCTCTG		
EIF2B2	Protein	NM_001015593.1	Reverse	CTTGAACATAGGAGCACAGACG	219	97.3
	synthesis		Forward	CATGAGATGGCAGTCAATTTGT		
GAPDH	Glucose	NM_001034034.1	Reverse	GAGCTTGACAAAGTGGTCGTTGAG	275	99.0
	metabolism		Forward	CCAACGTGTCTGTTGTGGATCTGA		
RNF20	Histone H3	NM_001081587.1	Reverse	GAGCCTGAAGAAGAGGTTCAAA	153	97.0
	methylation		Forward	TCCAGGTTCTCCTCGCTAATAC		
SF3A1	Splicing	NM_001081510.1	Reverse	ATTCCTGGTTTCACGTCTCCTA	194	96.6
	system		Forward	TGTGTCCCTCTTGCTGAGTTT		
UBE2D2	Protein	NM_001046496.1	Reverse	CTTCTCTGCTAGGAGGCAATGT	242	102.8
	degradation		Forward	TGGACTCAGAAGTATGCGATGT		

3.3 Results

3.3.1 Messenger RNA levels of reference genes

Efficiencies of reaction for candidate reference genes ranged from 93.4% to 102.8% (Table 3.2). The comparison of relative abundance (lower CT value = higher abundance) of mRNA levels of candidate reference genes (Fig. 3.1A) showed that GAPDH and ACTB were two most abundant, and EIF2B2 and RNF20 were two least abundant genes in granulosa cells ($P < 0.001$, data combined among follicle type, age, superstimulation status and processing). Messenger RNA levels of UBE2D2 were intermediate between ACTB and SF3A1.

Linear amplification of mRNA increased ($P < 0.005$) the level of transcripts (lower CT values by an average value of 1.3 cycles) of the candidate reference genes in granulosa cells compared to non-amplified samples (Fig. 3.1D; data combined among follicle type, age, super-stimulation status). Within the linear-amplified or non-amplified mRNA category, mRNA levels of candidate reference genes followed the similar trend (processing type*gene interaction, P=0.98).

The mRNA levels (mean CT ± Standard deviation) of the candidate reference genes, did not differ ($P > 0.05$) among age (aged vs. young cows, Fig. 3.1B), type of follicle (dominant vs. preovulatory follicle, Fig. 3.1C) or due to duration of FSH treatment (standard FSH duration vs. long FSH duration vs. FSH starvation, Fig. 3E).

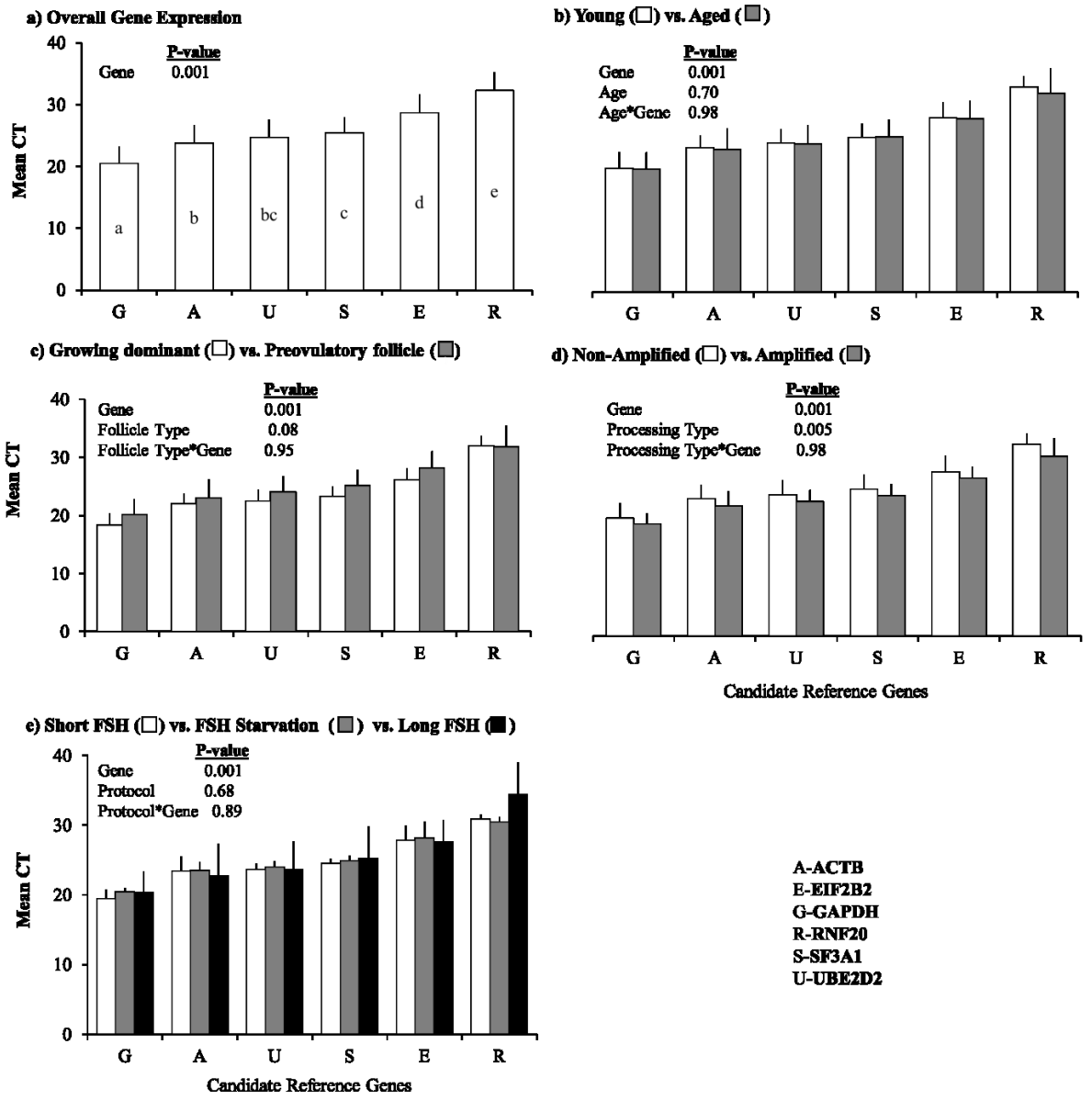

Figure 3.1 Comparison of expression levels (CT mean ± Std. dev.) of candidate reference genes (A, ACTB; E, EIF2B2; G, GAPDH; R-RNF20, S, SF3A1) in granulosa cells. Expression levels of candidate genes in a) combined dataset (data combined among age, follicle type**,** mRNA processing type and FSH protocol; open bars, n=19), b) young (open bars, n=5) versus aged (solid bars, n=6), c) growing dominant follicles (open bars, n=4) versus preovulatory follicles (solid bars, n=7), d) non-amplified (open bars, n=8) versus amplified (solid bars, n=11), e) short FSH duration (open bars, n=3) versus FSH starvation (solid bars, n=3) versus long FSH duration (dark bars, n=2). Different letters inside each bar in Fig. a. indicate statistical difference ($P < 0.05$). Factorial ANOVA *P*-values are indicated in each graph.

3.3.2 Stability of reference genes

The stability ranking order of the candidate reference genes differed among GeNorm, Delta CT and Normfinder for the granulosa cells of dominant follicles (n=11) or FSH-stimulated follicles (n=8) or in the combined dataset (n=19; dominant and FSH-stimulated follicles combined). EIF2B2 was ranked the most stable gene in the granulosa cells of dominant follicle by GeNorm (Fig. 3.2a) whereas UBE2D2 was ranked the most stable gene by NormFinder and Delta CT programs (Fig. 3.2d and g). GAPDH was ranked as the third most stable reference gene and RNF20 was ranked the least stable gene by all three programs. GeNorm, Delta CT and NormFinder ranked 2nd and 4th most stable genes differently (Fig. 3.2a, d, g). For granulosa cells of FSH-stimulated follicles, UBE2D2, EIF2B2 and SF3A1 were the three most stable reference genes ranked by GeNorm and Delta CT programs (Fig. 3.2 b, e) while this ranking order was reversed by NormFinder (Fig. 3.2i). GAPDH and ACTB were ranked either 4th or 5th by all programs. RNF20 was ranked the least stable gene by all three programs.

Overall (combined data from dominant and FSH-stimulated follicles), GeNorm, Delta CT and NormFinder ranked UBE2D2, EIF2B2, and SF3A1 as the three most stable genes followed by GAPDH and ACTB (Fig. 3.2c, f, i). RNF20 was ranked the least stable gene.

3.3.3 Comprehensive ranking order of reference genes

Considering the comprehensive ranking order, UBE2D2, EIF2B2, and GAPDH were the three most stable genes in the granulosa cells of dominant follicles, followed by ACTB and SF3A1 (Fig. 2J). EIF2B2, UBE2D2 and SF3A1 were the three most stable genes in the FSH-stimulated follicles (Fig. 3.2k) followed by GAPDH and ACTB. When data were combined among dominant and FSH-stimulated follicles (Fig. 3.2l), ranking order was identical to that for

FSH-stimulated follicles. RNF20 was the least stable gene in all three comprehensive ranking order analyses.

3.3.4 Optimal numbers of reference genes for determining normalization factor

Pair wise variation of the three genes (V_3/V_4; UBE2D2, EIF2B2 and GAPDH) or four genes (V_4/V_5; UBE2D2, EIF2B2, GAPDH and SF3A1) was below the cut off value of 0.15 (Fig. 3.3a) and geometric mean of these three or four genes was recommended to calculate the normalization factor. For granulosa cells of FSH-stimulated follicles, pair wise variation of two genes (V_2/V_3; UBE2D2 and EIF2B2) was below the cut off value of 0.15 (Fig. 3.3b) and geometric mean of these two genes was recommended to calculate normalization factor. For combined dataset (dominant and FSH stimulated follicles) pair wise variation of four genes (V_4/V_5; UBE2D2, EIF2B2, GAPDH and SF3A1) was below the cut off value of 0.15 and was recommended for calculation of NF by GeNorm (Fig. 3.3c).

Using the set of genes (UBE2D2, EIF2B2, GAPDH and SF3A1) from combined dataset (dominant + FSH-stimulated follicles), normalization factors were recalculated for dominant and FSH-stimulated follicle datasets (NF_4 recalculated) and correlated with normalization factors (based on minimum genes) of dominant or FSH-stimulated follicles. There was significant correlation ($P < 0.001$) between NF_4 (recalculated) vs. NF_3 (UBE2D2, EIF2B2 and GAPDH) of dominant follicles and between NF_4 (recalculated) vs. NF_2 (UBE2D2 and EIF2B2) of FSH-stimulated follicles. Therefore, a combination of genes (UBE2D2, EIF2B2, GAPDH and SF3A1) was more appropriate to calculate normalization factor for dominant or FSH-stimulated follicles or both categories.

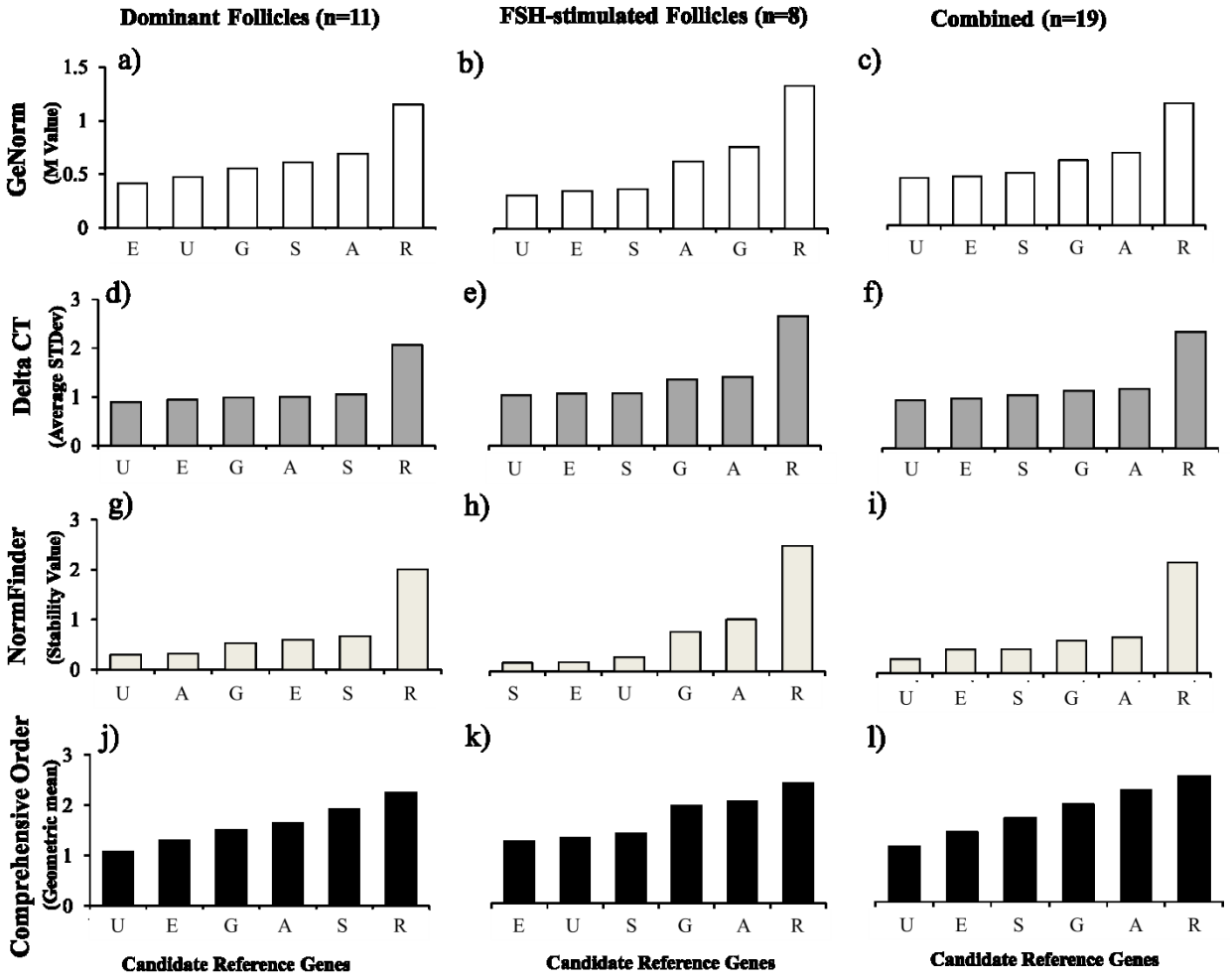

Figure 3.2 Ranking order of six candidate reference genes (A, ACTB; E, EIF2B2; G, GAPDH; R-RNF20, S, SF3A1) in granulosa cells. Genes were ranked based on M-value from GeNorm software (Fig. a, b, c), average standard deviation from Delta CT method (Fig. d, e, f), stability value from NormFinder software (Fig. g, h, i), and geometric mean from comprehensive ranking order method (Fig. j, k, l). Dominant follicles (Fig. a, d, g, j; n=11) and FSH-stimulated follicles (Fig. b, e, h, k; n=8) were analyzed separately or after combining the two datasets (Fig. c, f, i, l; n=19). Lower height of the bars represents higher stability value.

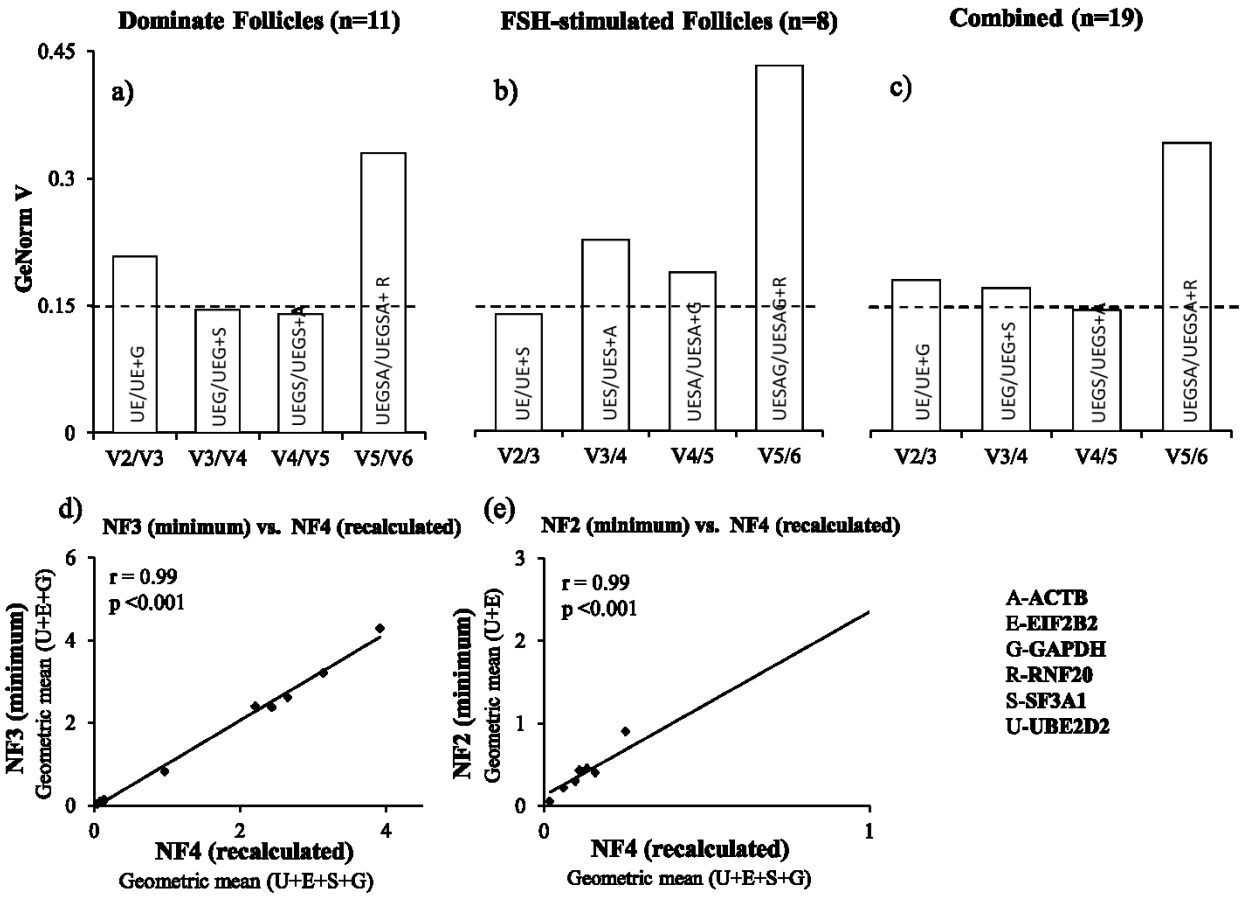

Figure 3.3 Optimal number of reference genes recommended by GeNorm for gene expression normalization of dominant follicles (Fig. a; n=11), FSH-stimulated follicles (Fig. b; n=8) or combined dataset (Fig. c; n=19). Dotted line represent cut off value of 0.15 and bars represents pairwise variation (V) between sequential normalization factors starting with minimum of two factors to maximum of six factors (V2 to V6). Label inside the bar represents combination of genes in pairwise comparison. A very high degree of correlation coefficient between recalculated normalization factor (NF4 recalculated) recommended by combined dataset analysis and the minimum original normalization factors for dominant follicles (Fig. d) and FSH-stimulated follicles (Fig. e) indicated that combination of four reference genes (UBE2D2, EIF2B2, GAPDH and SF3A1) is appropriate to normalize expression of genes of interest in studies involving dominant follicles alone, FSH-stimulated follicles alone or in studies that involve both categories of follicles. Each sample is represented by a data points along the correlation line in Fig. d and e.

3.4 Discussion

Incorrect selection and lower stability of reference genes can lead to erroneous measurement of mRNA levels of gene of interest (Haller *et al.*, 2004). Previous studies indicate that stability of two commonly used reference genes (GADPH and ACTB) may differ among tissue types, species or vary due to applied treatment (Suzuki *et al.*, 2000; Perez *et al.*, 2008). Since the stability of reference genes in bovine granulosa cells during maternal and follicular aging is not known, we evaluate the suitability of two commonly used (GADPH, ACTB) and four novel (UBE2D2, EIF2B2, SF3A1, RNF20) reference genes from dominant and FSH-stimulated follicles. Our results document that a combination of UBE2D2, EIF2B2, GAPDH and SF3A1 is more suitable method for relative gene expression studies of granulosa cells during maternal and/or follicular aging than use of individual reference genes.

Two common reference genes (GAPDH or ACTB) were selected based on previously published reports (Zielak *et al.*, 2008; Machado *et al.*, 2009). Four novel genes (UBE2D2, EIF2B2, SF3A1 and RNF20) were included based on their stability (least CV) in microarray data of dominant and preovulatory follicles from a maternal aging study. Other potential reference genes (HPRT (Ricken *et al.*, 2002), YWHAZ (Rodriguez *et al.*, 2007), UBC (Schmahl *et al.*, 2008), RPLO (Luo & Wiltbank, 2006), 18S rRNA (Voge *et al.*, 2004)) were also probed in the microarray dataset but were either more variable in their expression (≥1.5 fold change) or below the level of background noise in the microarray data. UBE2D2, EIF2B2, SF3A1 and RNF20 are commercially available as housekeeping genes and one gene (SF3A1) has been reported in bovine skeletal muscles as most stable reference gene (Perez *et al.*, 2008). In this study, number of tested reference genes was restricted to six due to limited volume of the samples available for testing.

The mRNA levels of GAPDH or ACTB in granulosa cells of dominant follicles were relatively consistent during maternal aging or follicular aging. However, based on the comprehensive ranking analyses, GAPDH and ACTB were considered less stable than UBE2D2 and EIF2B2. Lower stability of GAPDH or ACTB was also apparent when data from young and aged groups were analyzed separately (Data not shown). Likewise, GAPDH and ACTB were ranked at 4^{th} and 5^{th} place (among the six studied genes) in granulosa cells from FSH-stimulated follicles. These findings complement the earlier reports that indicated variable stability of GAPDH or ACTB as a result of aging in rat liver, human peripheral blood mono-nuclear cells and porcine tissues (Chen *et al.*, 2006; Zampieri *et al.*, 2010; Uddin *et al.*, 2011). Likewise, Gonadotropin-induced granulosa cell proliferation (Gilbert *et al.*, 2012) is known to modulate GAPDH levels in humans (Mansur *et al.*, 1993) and gonadotropin treatment of granulosa cells has been associated with increased ACTB levels in rats (Delidow *et al.*, 1990). Therefore, the use of GAPDH or ACTB as a single reference gene may not be the best choice for comparing the gene expression in granulosa cell from bovine dominant follicles.

The difference ($P < 0.005$) in transcripts of reference genes was not surprising because of mRNA processing type (amplified vs. non-amplified; Fig. 3.2). It has been demonstrated in previous report that T7 RNA polymerase linear amplification technique increases the gene transcripts in samples without change in size distribution of parent cDNA strand (Van Gelder *et al.*, 1990). There was also no significant interaction found between the genes and processing type ($P=0.98$) suggesting that the amplification did not affect the relative amount of gene transcripts between the two mRNA processing types and gene transcripts are parallel in both processing types.

Our results document that multiple reference genes are more appropriate than using a single reference gene for normalization of gene of interest. A combination of minimum of two and maximum of four reference genes is recommended by GeNorm for the calculation of normalization factor (Fig. 3.3). These findings support the previous reports suggesting the use of multiple reference genes (Suzuki *et al.*, 2000; Vandesompele *et al.*, 2002). It has been documented that normalization based on single reference gene can result erroneous measurement of mRNA levels of gene of interest by three to six-fold (Vandesompele *et al.*, 2002). Therefore, geometric mean of multiple reference genes is considered a preferable strategy to normalize the expression of gene of interest. In this study, high Pearson's coefficient correlations between NF_4 (recalculated) vs. NF_3 of dominant follicles or NF_2 of FSH-stimulated follicles indicates that a single set of genes can be used to calculate normalization factor for gene expression analyses of dominant and FSH-simulated follicles (separate and combined). This approach of correlation between normalization factors has previously been reported (Uddin *et al.*, 2011) and found useful when inclusion of multiple reference gene is not feasible due to limited sample volume. Based on results of this study, use of geometric mean of four genes, UBE2D2, EIF2B2, GAPDH and SF3A1, is recommended for normalization of relative gene expression in granulosa cells of dominant and FSH-stimulated follicles.

We used three programs (GeNorm, Comaparative delta CT and Normfinder) to analyze the stability of the reference genes. Information from these programs may be confusing owing to different approaches adopted by each program to calculate the stability value. Therefore, comprehensive ranking order of the reference genes was implemented as suggested elsewhere (http://www.leonxie.com/referencegene.php) - effectively eliminating the bias that could have arisen from use of a single program. The comprehensive ranking order for granulosa cells of

dominant and FSH stimulated follicles (Fig. 3.2) corresponded very well with the combination of reference genes (UBE2D2, EIF2B2, GAPDH and SF3A1) suggested for calculating the normalizing factor by individual programs.

This study systematically evaluated the stability of mRNA of six genes (GAPDH, ACTB, UBE2D2, EIF2B2, SF3A1 and RNF20) in granulosa cells of dominant follicles and FSH-stimulated follicles to select reference genes for normalizing relative gene expression during maternal and follicular aging. Reference genes were objectively ranked for their stability without exclusively relying on a single program. Although mRNA levels of GAPDH and ACTB were higher than other tested genes in granulosa cells of dominant or FSH-stimulated follicles, both were ranked less stable than UBE2D2 and EIF2B2 by comprehensive ranking. Use of geometric mean of multiple genes (UBE2D2, EIF2B2, GAPDH and SF3A1) is a better reference than the use of single gene for normalization of gene expression.

4 CHAPTER 4: MATERNAL AGE RELATED CHANGES IN TRANSCRIPTOME OF BOVINE GRANULOSA CELLS OF DOMINANT FOLLICLE AT THE TIME OF SELECTION

Muhammad Irfan-ur-Rehman Khan, Fernanda Caminah Faustino Dias, Marc-Andre Sirard, Gregg P. Adams and Jaswant Singh

Roles and Contributions of Co-authors: Fernanda Dias helped in tissue collection, sample processing and setting up RT-qPCR. Marc-Andre helped in data analysis and critically reviewed the manuscript; Gregg Adams helped in tissue collection, study design and critically reviewed the manuscript, and Jaswant Singh supervised, helped in study design, data analysis and critically reviewed the manuscript.

Relationship of this study to the dissertation

In mono ovulatory species, the selection of a dominant follicle from the cohort of antral follicles is a critical event. Any malfunction arising at this stage of follicle development may affect further development of the ovulatory follicle. In this context, transcriptome analysis of granulosa cells of the dominant follicle at the time of selection can reveal the developmental potential of the follicle before reaching preovulatory stage. Therefore, this study determines the changes in the transcriptome of the granulosa cells to understand the pathways and molecular functions that are compromised due to maternal age.

4.1 Introduction

An age-associated decline in fertility is well documented in animals and humans (Erickson *et al.*, 1976; Klein & Sauer, 2001). Pregnancy rates decline in women over 35 years of age; i.e., a healthy 30 years old woman has 20% chance of getting pregnant per cycle whereas a 40 years old woman has only 5% chance of getting pregnant per cycle (ASRM, 2012). Similarly, live birth rates after in vitro fertilization treatment decline from 37% in women at < 35 years of age, to 27% during 35-39 years of age and 11% at age > 40 years (Gunby & Daya, 2006). However, for ethical and practical reasons, human oocytes and ovarian tissues cannot be obtained for research and hypothesis testing. Therefore, these observations require further validation to understand underlying mechanism of reproductive aging and to improve outcome of assisted reproductive techniques. Studies done in senescence-accelerated mouse models and postovulatory aging of mouse oocytes (Liu *et al.*, 2002; Warner & Sierra, 2003; Liu *et al.*, 2004; Thouas *et al.*, 2005; Tatone *et al.*, 2006) have provided insights into molecular mechanisms of aging, but follicular control and ovulation mechanisms differ greatly between monovular and polyovular species (follicular waves, selection, dominance), making extrapolation to humans (Aviv, 2004) and domestic animals of limited value. Our research group has validated the use of bovine model (Adams & Pierson, 1995) to study the maternal aging (Malhi *et al.*, 2005; Malhi *et al.*, 2006) because follicular dynamics and hormonal control of follicular waves are well characterized in this species. Elevated circulating concentrations of FSH and reduced ovarian reserve in old cows (13-15 years) vs. young daughters (3-5 years) were similar to that reported in women approaching menopause (Malhi *et al.*, 2005). Further, pituitary and ovarian responsiveness to follicular wave synchronization treatment was similar between old cows and their young daughters, but aging was associated with a delay in the preovulatory LH surge

(Malhi *et al.*, 2006). Maternal aging in cattle was associated with a decline in follicular and ovulatory response after superstimulatory treatment (Malhi *et al.*, 2008). Oocytes from the old cows are markedly less competent; only 29% of oocytes from old cows produced blastocysts in vivo compared with 62% from their daughters (Malhi *et al.*, 2007).

Granulosa cells have been used as biomarkers to predict follicle health, oocyte quality and competence (Armstrong, 2001; Hamel *et al.*, 2008; Ito *et al.*, 2008; Gilbert *et al.*, 2012). Changes in the ultrastructure of mitochondria and endoplasmic reticulum of granulosa cells were noticed in the resting follicle pool in aged women (de Bruin *et al.*, 2004). Transcriptome analyses of granulosa cells at the time of dominant follicle selection (Evans *et al.*, 2004; Fayad *et al.*, 2004; Sisco & Pfeffer, 2007; Mihm *et al.*, 2008; Skinner *et al.*, 2008; Liu *et al.*, 2009), atresia (Tilly *et al.*, 1992), preovulatory maturation (Martoriati & Gerard, 2003; Tetsuka *et al.*, 2010) and luteinization (Wu & Wiltbank, 2002; Gilbert *et al.*, 2011) underscore the role of granulosa cells during folliculogenesis and attainment of developmental competence by the contained oocyte. However, there is limited information available about age-associated changes in granulosa cells (Ito *et al.*, 2008; Hurwitz *et al.*, 2010; Ito *et al.*, 2010). In previous studies of granulosa cells from aged vs. young women, interleukin family gene expression was lower (Hurwitz *et al.*, 2010), whereas glutathione S transferase-1 (Ito *et al.*, 2008) and p38 MAPK proteins expressions were higher (Ito *et al.*, 2010). It is noteworthy that the granulosa cells used in these studies were obtained from super-stimulated follicles; i.e., administration of gonadotropins removed the natural process of follicle selection. To understand the underlying molecular basis of age-related changes in follicular function and endocrine milieu, it is important to examine the differences in mitotic ability, apoptosis, responsiveness to gonadotropins, oxidative status and steroidogenic pathways in granulosa cells during the process of selection of

the dominant follicle (i.e., period during which dominant follicle continue to grow while the subordinate follicles of the cohort undergo regression).

The objective of the present study was to determine the effect of maternal age on the transcriptome of bovine granulosa cells collected at the time of selection of the dominant follicle to undercover the changes in molecular and cellular functions of the granulosa cells. Extension of the bovine model of maternal aging to cellular and molecular level will allow proposing new hypotheses to explain follicle–associated loss of oocyte competence in aged cows.

4.2 Materials and methods

4.2.1 Animals and tissue collection

Granulosa cells were collected from aged (n=5, Mean ± SEM, 15 ± 1.5 years) and young cows (n=6, 7 ± 0.8 years) after ultrasound-guided follicle aspiration or ovariectomy. Aged and young cows were Hereford-cross beef cattle maintained together in a single corral at the University of Saskatchewan Goodale Farm. They were born on this farm so their birth records and calving history records were available. The cows had not been pregnant nor lactated in the previous two years. Four young cows were daughters of the aged cows. Due to limited number of animals, follicle collections were performed twice from 3 animals, i.e. 14 follicles were collected from 11 animals. From these cows, granulosa cells for RT-qPCR analysis were obtained first, followed by surgical removal of ovary at a later time. Minimum time interval between follicle aspiration and ovariectomy was of two months. Nine follicles were collected by ovariectomy (n=5 follicles in aged and n= 4 follicles in young cows) and 5 follicles were collected by ultrasound-guided follicle aprirations (n=2 follicles in aged and n=3 follicles in young cows).

For microarray analyses a total of six follicles (3 per age group) were used. Out of the six follicles, 3 follicles (n=2 in old and n=1 in young cow) were obtained by ultrasound guided

follicle aspiration and remaining 3-follicles (n=1 in old and n=2 in young cows) were obtained after ovariectomy at later date. All young cows (n=3) used for microarray analysis were daughters of the old cows (n=3). Microarray results were validated by real-time quantitative polymerase chain reaction (RT-qPCR) using granulosa cells from 8 dominant follicles (n=4 per age group). Six follicles eight dominant follicles (n=3-follicles per age group) were aspirated by ultrasound guided follicle puncture whereas remaining 2-follicles (n=1 per age group) were collected after ovariectomy. A total of two cows (one old and one young) were same between the microarray and RT-qPCR studies but did not share the cells from a given dominant follicle. The experimental protocol was approved by the University of Saskatchewan Committee on Animal Care and procedures were conducted in compliance with the guidelines of the Canadian Council on Animal Care.

Before tissue collection (ovariectomy or transvaginal follicle aspiration), cows were given a luteolytic dose of prostaglandin ($PGF_{2\alpha}$; Lutalyse, 25 mg i.m., Pfizer Animal Health, Kirkland, QC, Canada) to synchronize ovulation. The ovaries were examined daily by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SD 900, Tokyo, Japan) to detect ovulation. The day of ovulation was taken as the day of emergence of the first follicular wave (Day 0) (Singh *et al.*, 1997; Malhi *et al.*, 2005). Granulosa cells were harvested on Day 3 (i.e., by ovariectomy or transvaginal aspiration).

Transvaginal ultrasound-guided follicle aspiration was done using a 5 MHz convex-array transducer (Aloka SSD 900, Tokyo, Japan) equipped with a disposable needle (catalog # 305833, 18 ga x 1.5”; BD Medical, Mississauga, ON, Canada) connected to a 10 ml syringe by silicone tubing (115 cm long, 1.14 mm internal diameter, Cole-Palmer, Montreal, QC, Canada), as described (Berfelt *et al.*, 1994). Caudal epidural anesthesia was induced, and perineum was

disinfected as described above. After introducing the transducer into the vaginal fornix, the ovary was positioned against transducer face by transrectal manipulation, and the dominant follicle was punctured and follicular contents were aspirated. The cumulus-oocyte-complex (COC) was identified under a stereomicroscope and separated from the aspirate. The follicular fluid was centrifuged at 700g for 15 minutes to harvest the pellet of antral granulosa cells.

Unilateral ovariectomies were performed by colpotomy, as described (Singh *et al.*, 1998). Briefly, caudal epidural anesthesia was induced with 5 to 10 ml of lidocaine HCl 2% (catalog # 1LID009P; Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). The perineum was disinfected using an iodine-based detergent and solution. A small incision was made in the dorso-lateral aspect of the vaginal fornix. The peritoneum was ruptured manually, allowing direct access to and palpation of the reproductive tract. Local anesthesia was applied to ovarian pedicle using a gauze soaked with lidocaine. The chain of an ecraseur (Jorgensen Labs, catalog # J0037E, Loveland, Colorado, USA) was looped around the ovarian pedicle and slowly tightened until the ovarian attachments were severed. The cows were treated with procaine penicillin G (Pen G injection; 21000 IU/Kg, i.m., Citadel Animal Health, Edmonton, AB, Canada) daily for four days after ovariectomy.

The ovary was placed in a polyethylene bags, kept on ice, and transported to the laboratory within 5 minutes after collection. The dominant follicle and the largest subordinate follicle (if located ipsilateral to the dominant follicle) were identified based on ultrasonographic records and confirmed by measuring the diameter after follicles were opened. The goal was to collect antral and mural granulosa cells of the dominant follicle. Antral granulosa cells were collected by aspiration of the follicular antrum using a 20 gauge needle and syringe. Follicles were flushed 3-times with Delbecco's phosphate buffer saline (DPBS, catalog # 21600-010; Life

Technologies, Burlington, ON, Canada). The cumulus-oocyte-complex (COC) was identified and separated from the aspirate. The follicular fluid was centrifuged at 700g for 15 minutes to harvest the pellet of antral granulosa cells. The collapsed follicles were opened in half using a scalpel blade, and the inner follicular wall was scraped using a microbiology culture-loop (catalog # PD104; LightLabs, Dallas, TX, USA) to remove the mural layer of granulosa cells. The mural and antral granulosa cells were kept separate and were either snap frozen in liquid nitrogen and stored at -80 °C or suspended in 300-700 µl of RNA stabilization and protection solution (RNA*later*, catalog #. AM7020; Life Technologies, Burlington, ON, Canada) for 12 h at 4 °C before storing at -80 °C for later microarray analysis. However, only antral granulosa cells were used in this study.

4.2.2 Concentrations of estradiol and progesterone in follicular fluid

Radioimmunoassays were used to measure estradiol 17β and progesterone concentrations in the follicular fluid of the dominant (n=7 per group) and largest subordinate follicles (3-4 per group) from aged vs. young cows, as described (Singh *et al.*, 1998). Briefly, charcoal-extracted pooled bovine follicular fluid from abattoir-collected ovaries was used for preparation of the standards and dilution of the samples for estradiol 17β (1:100 or 1:500; v:v) or progesterone (1:40) assay. Commercially available radioimmunoassay kits were used for both estradiol (Kit # KE2D1, Coat-A-Count, Siemens Health Care Diagnostics Inc., Malvern, PA, USA) and progesterone (Kit # TKOP1, Coat-A-Count, Siemens Healthcare Diagnostics Inc.). The detection range of estradiol assay was 5-500 pg/ml while for progesterone assay was 0.1-40 ng/ml, respectively. Samples were analyzed in duplicate in single assay for each hormone. Intra-assay coefficients of variation for estradiol and progesterone assays were 11% and 2.5%, respectively.

4.2.3 Granulosa cell RNA extraction, amplification and labeling

Total RNA was extracted from granulosa cells digested with DNase 1 (catalog # 79254, Qiagen, Toronto, ON, Canada) and eluted in 15 µl of the buffer using RNA Isolation Kit (catalog # KIT0204; Life Technologies, Burlington, ON, Canada) as prescribed in the manufacturer's protocol. RNA integrity and concentration were determined using a 2100-Bioanalyzer, (Agilent Technologies, Santa Clara, CA, USA). For microarray analysis, linear amplification of 5 ng of total RNA was done by RNA amplification Kit (catalog # KIT0525, Life Technologies, Burlington, ON, Canada) according to the manufacturer's protocol. Labeling of 2 µg of antisense-RNA with Cy3 and Cy5 florescent dyes was done using ULS florescent labeling kit for Agilent arrays (Kit # EA-021, Keratech Biotechnology, Keratech Biotechnology, Amsterdam, The Netherlands) according to the manufacturer's protocol. Labeling efficiency for both dyes was measured by spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

4.2.4 Microarray design, hybridization, and scanning

For microarray analysis, three biological replicates (each replicate consisted of one aged and one young cow) were compared directly on three separate microarrays. The bovine oligo-array slide was custom-designed (EmbryoGENE EMBV3, Design ID: 028298, GEO accession # GPL13226; Agilent Technologies) and consisted four arrays of 37,351 genes or isoforms (excluding the control spots) in a 4x44K format (Robert *et al.*, 2011). Briefly, in each of three biological replicates, 825 ng of Cy3-labeled antisense-RNA from an aged cow and 825 ng of Cy5-labeled antisense-RNA from a young cow were mixed and hybridized on an array. To control for non-biological variation, each of the three biological replicates had a technical replicate; i.e. Cy3 and Cy5 dyes were reversed in a biological replicate (dye swap) and hybridized to a separate array (Robert *et al.*, 2011). In total, six hybridizations were performed

that included three biological and three technical replicates. Microarray slides were incubated at 65 °C for 17 h in a rotating oven (rotor speed, 10 rpm) and then washed using a gene expression wash buffer kit (catalog # 5188-5327, Agilent technologies, Burlington, ON, Canada). Briefly, slides were serially washed with "wash buffer 1" and "wash buffer 2" for 3 min at 42 ºC each, and dipped into acetonitrile (100%) for 10 sec at room temperature. Slides were then washed with stabilizing and drying solutions for 20 sec each at room temperature and scanned by a Power Scanner (Tecan Group Ltd. Mannedorf, Switzerland) for image acquisition. Images were quantitated for signal intensity using Array-Pro software (Media Cybernetics Inc., Rockville, MD, USA).

4.2.5 Data normalization and statistical analysis

Signal intensity data were uploaded to the EmbryoGENE Laboratory Information Management System and the microarray analysis platform in MIAME-compliant fashion (minimum information about microarray experiments; http://elma.embryogene.ca). The microarrays were assessed for linearity, specificity, and variability of hybridization (http://elma.embryogene.ca). Data files were imported into Flexarray software (version1.6.1) to normalize signal intensities and to generate a list of differentially expressed genes, as described (Michal Blazejczyk, 2007). Briefly, the signal intensity of the background of a target was subtracted from the median signal intensity of the foreground of the target. If the difference was a negative value due to higher intensity of the background vs. foreground signals, the negative value was replaced with a default value of 0.5. The median pixel value for each target was transformed to the $\log_2$ and normalized "within array" for dye bias using nonparametric regression (locally weighted scatter plot smoothing; "lowess"), and subjected to "between-array" normalization to unify intensities across the arrays. Finally, a list of differentially expressed

genes was obtained by running a simple linear model for microarray data; i.e., "simple limma algorithm" (Michal Blazejczyk, 2007). A gene was considered to be differentially expressed when a ≥ 2-fold change in its expression at *P*-value of ≤ 0.05, was detected. Benjimeni-Hocheberg method with a fold change of ≥ 2 and *P*-value of ≤ 0.05 was used to determine true positive genes. Data were deposited in NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) with a GEOseries accession number: GSE52480.

4.2.6 Functional analyses

Pathway analysis software (Ingenuity Pathway Analysis; www.ingenuity.com) was used to help understand the biological context of the differentially expressed genes (≥ 2-fold change*;* $P \leq 0.05$) in granulosa cells of aged cows vs. young cows. Genes that matched the Ingenuity knowledge base were processed for functional annotation, pathways, networks and upstream regulator analyses (Dias *et al.*, 2013).

4.2.7 Real time quantitative polymerase chain reaction (RT-qPCR)

To validate the microarray analyses, real-time qPCR analysis of six genes (*GJA1, PCNA, CYP19A1, VNN1, TPM2* and *INHBA*) was done using granulosa cells from aged and young cows collected by transvaginal ultrasound-guided follicle aspiration (n=4 cows per age group). Complimentary-DNA (cDNA) was synthesized by q-Script (Kit # 95047-100, Quanta Biosciences Inc.) from 50 ng of total RNA from granulosa cells of aged and young cows. A primer pair of each gene was designed by Primer3 (http://frodo.wi.mit.edu/primer3/) and analyzed for hairpins, secondary loops and compatibility using OligoAnalyzer 3.1 (Integrated DNA Technologies; www.scitools.idtdna.com/scitools/Applications/OligoAnalyzer). The specificity of each primer pair was confirmed using Basic Local Alignment Search Tool (BLAST) against the NCBI database (www.blast.ncbi.nlm.nih.gov/). The dissociation curve and

amplification efficiency of the primer pair of each gene was analyzed by MxPro software (www.genomics.agilent.com; Table 4.1). The amplicon of the primer pair of each gene was extracted on 1% agarose gel-electrophoresis (QIAquick gel extraction kit # 28704; Qiagen) and confirmed by sequencing the appropriate-size band using ABI 3730 XL DNA analyzer (Applied Biosystems). The quality of the sequenced amplicon was analyzed by Gap 4.4 software (Staden-Package; http://www2.mrc-lmb.cam.ac.uk/). The specificity of the amplicon was confirmed by BLAST. The purified and sequenced amplicon of each gene was used to prepare a standard curve ranging from 10^{-2} to 10^{-9} ng/µl to calculate the relative amounts of cDNA of a gene in the samples from aged and young cows.

A separate PCR was done for each gene, which included a standard curve, a no-template control (NTC) and cDNA of granulosa cells from aged cows and young cows. All granulosa cell cDNA samples were run in single reactions while the standard curve and NTC were run as multiple reactions. The PCR reaction mixture (total volume of 25 µl) contained 2 µl of cDNA of each sample, 12.5 µl of SYBR green master mix II (Kit # 600828; Agilent Technologies), 1875 nM of each forward and reverse primer, 0.375 µl of reference dye and 6 µl of nuclease-free water. Each PCR cycle was programmed for three-step amplification (i.e., denaturation, annealing, and extension) using Mx3005P machine (Agilent Technologies). Briefly, the first denaturation was done at 95 ºC for 10 min and then for 30 sec in the following 40 cycles to activate DNA polymerase and yield single strands of DNA. The annealing temperature for primers was kept constant (i.e., 55 ºC for 1 min) throughout the 40 cycles. Extension of the single strands of cDNA was done by DNA polymerase at 72 ºC for 1 min throughout the 40 cycles. For the analysis of RT-qPCR, the relative amount of each gene was normalized using the geometric mean of four reference genes (UBE2D2, EIF2B2, GAPDH and SF3A1) (Khan *et al.*,

2013). The normalized quantity of each gene was compared statistically between aged vs. young cows using REST software (Qiagen 2009) (Pfaffl *et al.*, 2002).

4.2.8 Statistical analyses for follicular and hormonal datasets

Statistical Analysis System software package (SAS version 9.2; SAS Institute Inc., Cary, USA) was used to analyze follicular diameter profiles and growth rate using repeated measures (PROC MIXED procedure). Dominant follicle data were compared for the main effects (age and day of wave) and their interactions (age*day). Dominant and subordinate within each age group were compared for follicle type, day and their interactions. Intrafollicular estradiol 17β and progesterone concentrations, and estradiol 17β to progesterone ratios of the dominant and the largest subordinate follicles were compared by two-way analysis of variance.

4.3 Results

4.3.1 Follicular and hormonal dynamics

At the time of selection of the dominant follicle of the first follicular wave (3 days after ovulation), no difference was detected in aged vs. young cows in dominant follicle diameter, growth rate (Fig. 4.1), intrafollicular estradiol 17β concentration or estradiol 17β: progesterone ratio (Table 4.2). Intrafollicular concentrations of progesterone in dominant follicles and estradiol 17β in largest subordinates was lower ($P < 0.05$) in aged cows compared to young cows. Within age group, the diameter and estrogen: progesterone ratio of the dominant vs. largest subordinate follicle differed ($P < 0.05$), confirming the anticipated status of the dominant follicles used in this study.

4.3.2 Differential gene expression

Of the 37,351 genes or isoforms tested in the granulosa cells of aged vs. young cows, 169 genes or isoforms were expressed differentially at a ≥ 2-fold change and *P*-value ≤ 0.05. In aged cows relative to young cows, 62 genes were up-regulated, and 107 genes were down-regulated (Fig. 4.2). The top ten up- and down-regulated transcripts are shown in Table 4.3.

4.3.3 Functional classification of transcripts

Among 169 differentially expressed genes or isoforms, 104 were annotated for functional classification by pathway analysis software. Analysis of molecular and cellular functions revealed that those most affected by age (*P*-value range from 2.05 $x10^{-5}$ to 2.67 $x10^{-5}$) were related to the cell cycle, DNA replication or recombination and repair, lipid metabolism, and small molecule transport (Fig. 4.3A). Canonical pathways that were most affected by age (*P*-value range from 0.004 to 0.03) were nitrogen metabolism, lysine biosynthesis, LXR/RXR activation, p53 signaling and cell cycle checkpoint control (Fig. 4.3B).

4.3.4 Upstream regulators

Based on expression of genes in the dataset, pathway analysis generated a list of potentially "inhibited" and "activated" upstream regulators (Table 4.4) that can explain the observed gene expression changes in the granulosa cells. Potential upstream regulators were included in the network as core molecules to explain downstream changes in genes expression (Fig. 4.4). About 70% of the upstream molecules were "inhibited" (i.e., their influence on downstream functions was predicted to be decreased), including FSH, estrogen, epidermal growth factor (EGF), sterol regulatory element binding transcription factor 1 and 2 (*SREBF-1* &

2). Potentially "activated" upstream regulators included tumor protein p53 (*TP53*) and mitogen-activated protein kinase 14 or protein 38 (*MAPK14 or MAPK p38*).

Table 4.1 List of primers used for RT-qPCR analysis to validate microarrays. The genes and details of primers along with expected amplicon size and obtained PCR reaction efficiency are provided.

Gene	Accession Number	Oligo	Primer pair Sequence (5' to 3')	Amplicon size (bp)	Efficiency (%)
Target Genes					
PCNA	NM_001034494.1	Forward	TCTCAGTCACATTGGAGATGCT	221	101.4
		Reverse	TAGGAGACAGTGGAGTGGCTTT		
GJA1	NM_174068.2	Forward	ACAAATCCTTCCCAATCTCTCA	230	100.0
		Reverse	GTGCTCTTCAATGCCATACTTG		
CYP19A1	NM_174305.1	Forward	CTCAATACCAGGTCCCAGCTAC	232	105.7
		Reverse	CAACCCAAGTTTACTGCCAAAT		
INHBA	NM_174363.2	Forward	CCAAAGGATGTACCCAACTCTC	196	96.5
		Reverse	GTCCGATGTCGTCCTCTATCTC		
VNN1	NM_001024556.2	Forward	TATTCTCTTCCACGATCCTGCT	197	105.3
		Reverse	TTCCACTCCCTGTCATTTTCTT		
TPM2	NM_001010995.2	Forward	GAGATCACCCACAAAACACTGA	234	101.5
		Reverse	CATCCCTCTCCTACACTTCACC		
Housekeeping Genes					
EIF2B2	NM_001015593.1	Forward	CATGAGATGGCAGTCAATTTGT	219	97.3
		Reverse	CTTGAACATAGGAGCACAGACG		
GAPDH	NM_001034034.1	Forward	CCAACGTGTCTGTTGTGGATCTGA	275	99.0
		Reverse	GAGCTTGACAAAGTGGTCGTTGAG		
SF3A1	NM_001081510.1	Forward	TGTGTCCCTCTTGCTGAGTTT	194	96.6
		Reverse	ATTCCTGGTTTCACGTCTCCTA		
UBE2D2	NM_001046496.1	Forward	TGGACTCAGAAGTATGCGATGT	242	102.8
		Reverse	CTTCTCTGCTAGGAGGCAATGT		

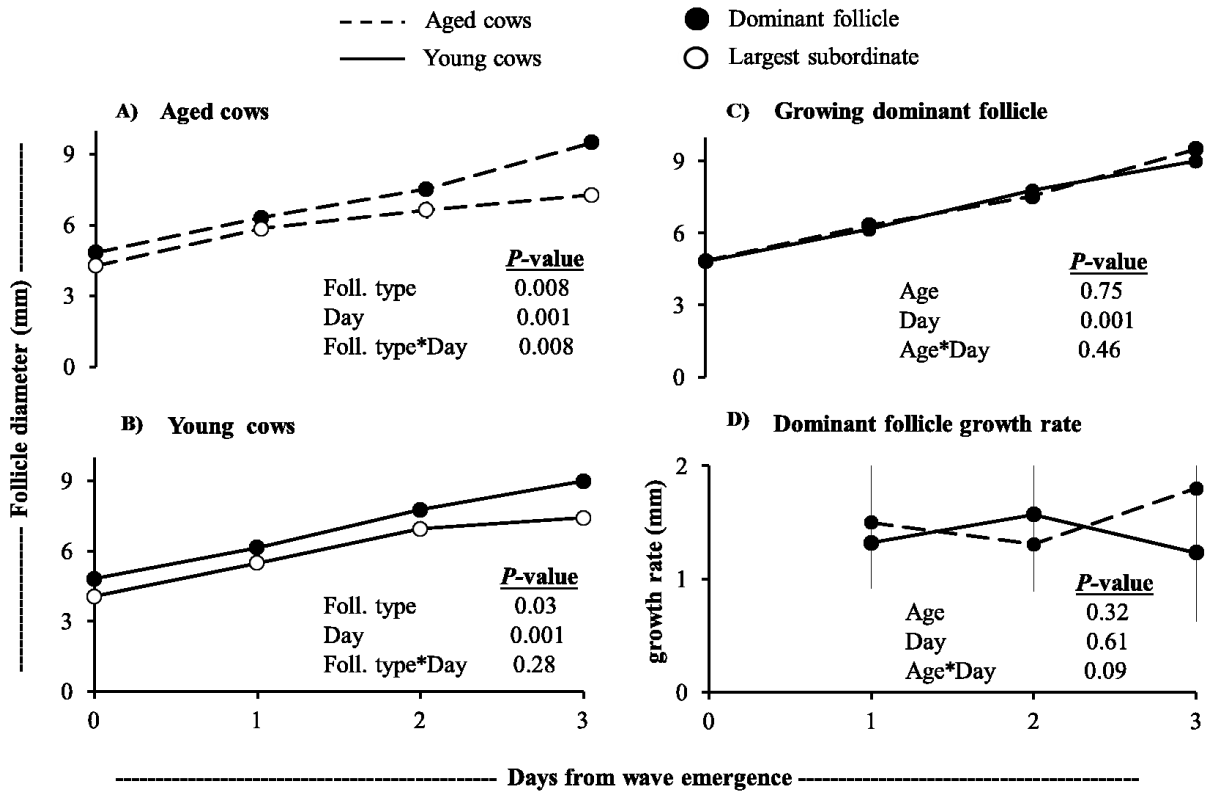

Figure 4.1 Comparison of diameters and growth rate (mm) of ovarian follicles of aged (n=5; total of 7 records of follicular waves) and young cows (n=6; total of 7 records of follicular waves). A) Growing dominant follicle vs. largest subordinate follicle from aged cows. B) Growing dominant follicle vs. largest subordinate follicle from young cows. C) Growing dominant follicle (aged cows vs. young cows). D) Growth rate of growing dominant follicle (aged cows vs. young cows). Diameter and growth rate of ovarian follicles are aligned to the day of new follicle wave emergence (day of ovulation). *P*-values for the age, follicle type, day and interactions (age*day) and (follicle type*day) are shown in graphs (SAS Proc Mix procedure).

Table 4.2 Intrafollicular concentrations of estradiol 17β, progesterone, and their ratio (mean ± SEM) in the dominant and largest subordinate follicles at the time of selection (3-days after follicular wave emergence) in aged and young cows. The dominant follicles are estrogenic while largest subordinates do not produce estradiol at this time.

Group	Follicle type	Estradiol 17β (ng/ml)	Progesterone (ng/ml)	Estradiol: Progesterone ratio
Aged	Dominant (n=7)	130.98 ± 56.09^{a}	21.12 ± 4.27^{a}	5.22 ± 1.31^{a}
	Subordinate (n=3)	0.34 ± 0.03^{b}	20.9 ± 4.41^{a}	0.02 ± 0.00^{b}
Young	Dominant (n=7)	152.84 ± 50.38^{a}	37.54 ± 4.07^{b}	4.07 ± 1.20^{a}
	Subordinate (n=4)	0.04 ± 0.03^{c}	14.31 ± 5.09^{a}	0.00 ± 0.00^{b}

[abc] Within columns, values with different superscript are different ($P < 0.05$).

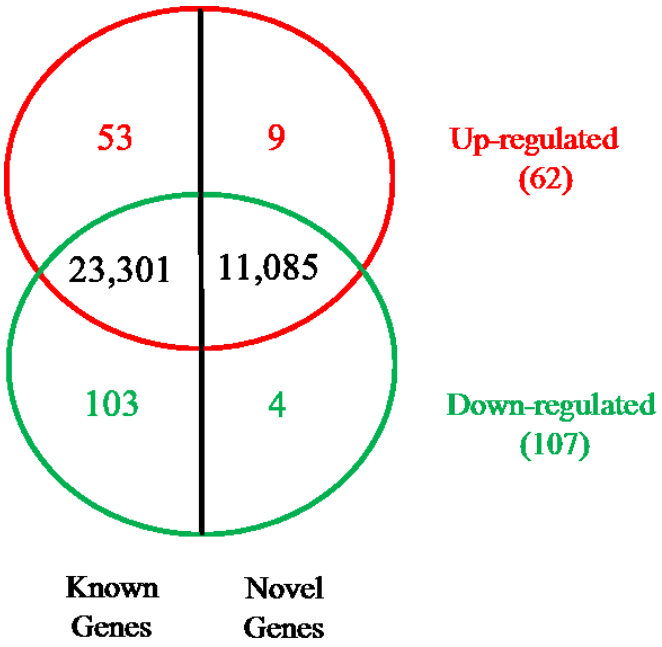

Figure 4.2 Venn diagram representing the number of transcripts up-regulated (red, upper circle) and down-regulated (green, lower circle) in granulosa cells from dominant follicles of aged vs. young cows. The overlapping region (center) represents transcripts that are common to both aged and young cows. The left half of each circle represents the number of known genes and right half represents novel genes. A total of 169 transcripts were differentially expressed in aged cows vs. young cows using a threshold value of ≥ 2-fold change and $P < 0.05$.

Table 4.3 The top 10 up-regulated and down-regulated genes at fold change ≥ 2; $P \leq 0.05$ in granulosa cells from dominant follicles of aged vs. young cows, as assessed by microarray.

Expression	Gene symbol	Protein encoded	Fold change
Up-regulated	VNN1	Vanin 1 or Vascular non-inflammatory molecule 1	5.5
	VNN2	Vanin 2 or Vascular non-inflammatory molecule 2	3.8
	MAN1A1	Mannosidase, alpha, class 1A, member 1	3.6
	MXRA8	Matrix-remodelling associated protein 8	3.6
	RPL36	Ribosomal protein L36	2.9
	LRRC17	Leucine rich repeat containing 17	2.8
	ANGPT2	Angiopoietin 2	2.8
	GRM8	Glutamate receptor, metabotropic 8	2.8
	RCBT2	Regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2	2.7
	CNKSR3	Connector enhancer of kinase suppressor of ras 3	2.6
Down-regulated	BEX2	Brain expressed X-linked 2	-4.6
	CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	-4.4
	IDH3A	Isocitrate dehydrogenase 3 (NAD+) alpha	-4.3
	TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	-3.5
	SLC39A14	Solute carrier family 39 (zinc transporter), member 14	-3.3
	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	-3.2
	LRP8	Low density lipoprotein receptor-related protein 8,	-3.2
	GJA1	Gap junction protein, alpha 1, 43kDa	-3.2
	SLC39A8	Solute carrier family 39 (zinc transporter), member 8	-3.2
	LDLR	Low density lipoprotein receptor	-2.9

4.3.5 Network analysis

To determine the relationships among differentially expressed genes, several networks were generated by pathway analysis software. The most significant network (highest Score = 52; Fig. 4.4) revealed that almost all the differentially expressed genes were down-regulated in aged cows, and were related to the control of 1) gonadotropins (INHBA, INHBB, FST), 2) the cytoskeleton (TNFAIP6, VCAN and beta tubulins) and extracellular matrix (TPM2, ACTG2), 3) lipid or cholesterol metabolism (LDLR, SCD, HMGCS1) and steroidogenesis (CYP19A1, CYP51A1), 4) cellular proliferation, replication and DNA repair (PCNA), intercellular communication (GJA1), and 5) increased oxidative stress due to up-regulated expression of genes (VNN1, GPX3). Information about other relevant genes has been provided in Appendix-A as Supplementary Table 10.1.

4.3.6 Validation of differentially expressed transcripts via RT-qPCR

Five of six transcripts analyzed by RT-qPCR had the same expression pattern in aged vs. young cows (Fig. 4.5) as detected in the microarrays (VNN1; $P \leq 0.05$), (CYP19A1; $P \leq 0.1$), (PCNA; $P \leq 0.01$), (TPM2; $P \leq 0.1$) and (GJA1; $P \leq 0.05$). Expression values of one transcript (INHBA) did not reach statistical significance but had a trend of decreased expression similar to the expression in the microarray.

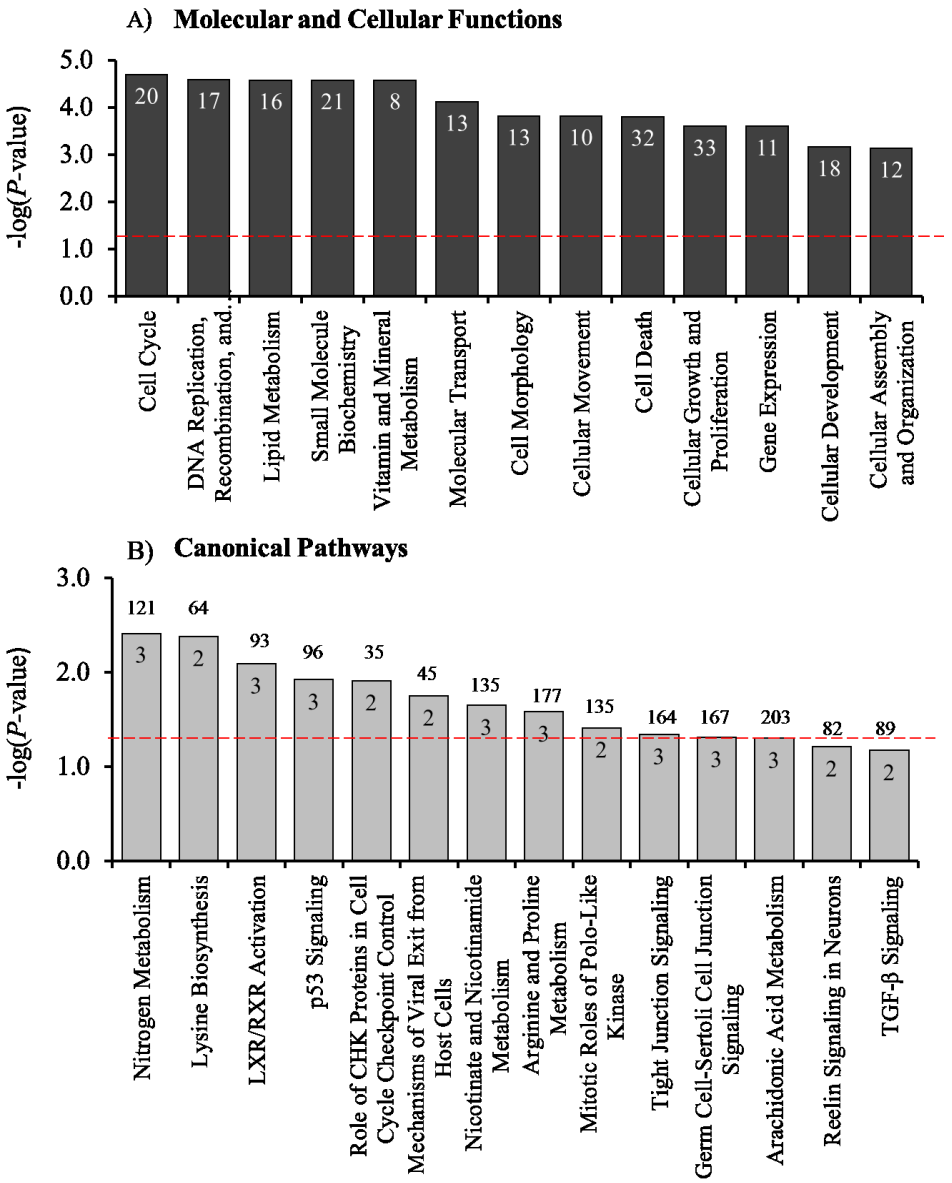

Figure 4.3 Annotation of differentially expressed gene of granulosa cells from aged vs. young cows with molecular and cellular functions (A), and canonical pathways (B) at the time of dominant follicle selection. The number inside each bar represents the number of transcripts annotated to the given function or pathway. The number at the outside of each bar in the canonical pathway (B) represents the total number of transcripts known to be associated with that specific pathway. The horizontal axis represents various functions (A) and pathways (B) that are most affected by age. The vertical axis represents the level of significance in the degree of differential gene expression in aged vs. young cows at or above the threshold value of $P \leq 0.05$ (equal to –log 1.30; red dotted line). A taller bar represents a greater level of statistical significance. Graphs generated by Ingenuity Pathway Analysis software.

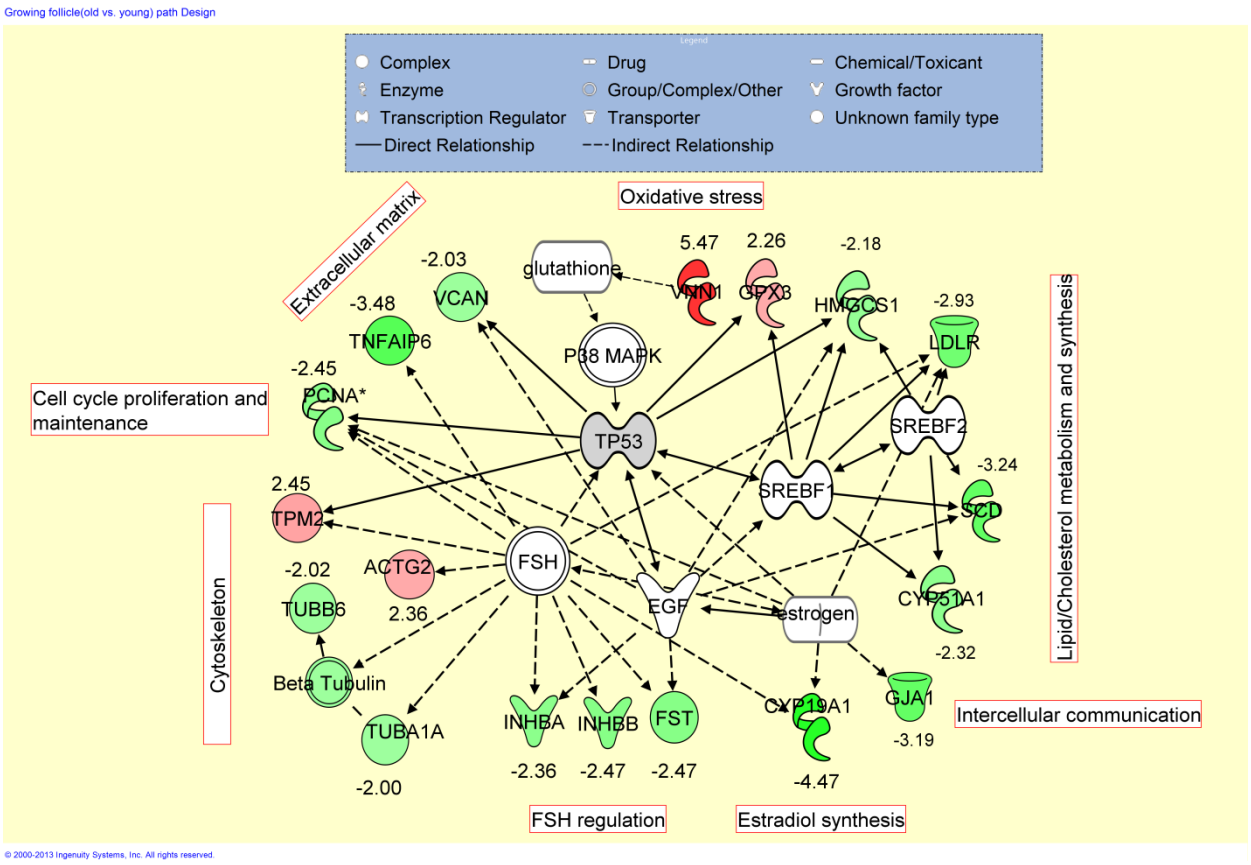

Figure 4.4 Network of genes (represented as nodes) and the biological relationship between genes (lines) differentially expressed in granulosa cells of growing dominant follicles from aged vs. young cows. Upstream regulators (EGF, Estrogen, FSH, p38MAPK (same as MAPK14), SREBF-1, SREBF-2, TP53) were identified by the Ingenuity Pathway Analysis software (Table 4) and were added to the network. Relationship-lines are supported by at least one reference derived from the literature, textbook, and/or canonical information stored in the Ingenuity Knowledge Base. The intensity of the color for each node indicates the degree of up (red)- or down (green)- regulation of a gene expression based on the fold change (shown next to each gene) in aged cows relative to young cows. Genes that were not differentially expressed or the difference was ≤ 1.5-fold are shown as white or grey, respectively. The shape of each node represents the functional class of the gene product (see legend) and is clustered with other genes based on the cellular functions (labels). Gene symbols are extracted from Entrez Gene or UniGene. Illustration generated by Ingenuity Pathway Analysis software.

Table 4.4 Upstream regulators predicted by pathway analysis software to be activated (increased influence on downstream targets) or inhibited (decreased influence) based on expression of target molecules identified in granulosa cell of the dominant follicle from aged vs. young cows. Up- and down-ward arrows indicate up-regulated and down-regulated expression of the transcripts, respectively.

Upstream regulator	Molecule type	Predicted action	Activation Score*	*P*-value	Target molecules in the dataset
TP53	Transcription regulator	Activated	2.249	$4.73x10^{-9}$	↑ASNS, ↓CHEK1, ↑CHMP4C, ↑GPX3, ↓H2AFZ, ↓LDHA, ↓HMGCS1, ↓ID2, ↓INHBA, ↑IRF7, ↑LRRC17, ↓MAD2L1, ↓MCM4, ↓NUSAP1, ↓PBK, ↓PCNA, ↓PTTG1, ↓SLC6A1, ↓TFDP1, ↓TP53BP2,TPM2, ↓VCAN
MAPK14	Kinase	Activated	2.000	$7.25x10^{-4}$	↓CYP51A1, ↓GJA1, ↓LDLR, ↑MYH11, ↑TPM2
EGF	Growth Factor	Inhibited	-3.407	$1.03x10^{-7}$	↓FST, ↓GJA1, ↓HMGCS1, ↓ID2, ↓IDI1, ↓INHBA, ↓LDHA, ↓MAD2L1, ↓PBK, ↓PCNA, ↓PTTG1, ↓SCD, ↓TFDP1
SREBF1	Transcription regulator	Inhibited	-2.380	$1.47x10^{-7}$	↓CYP51A1, ↑GPX3, ↓HMGCS1, ↓HSPA5, ↓IDI1, ↓LDLR, ↓MSMO1, ↓OAT, ↓SCD
SREBF2	Transcription regulator	Inhibited	-2.359	$1.38x10^{-6}$	↓CYP51A1, ↓HMGCS1, ↓IDI1, ↓LDLR, ↓MSMO1, ↓SCD
FSH	Complex	Inhibited	-2.322	$2.77x10^{-6}$	↑ACTG2, ↓CYP19A1, ↓FST, ↓INHBA, ↓INHBB, ↓LDLR, ↓MSMO1, ↓PCNA, ↓TNFAIP6, ↑TPM2, ↓TUBA1A
Estrogen	Chemical Drug	Inhibited	-2.604	$3.43x10^{-4}$	↓CYP19A1, ↓GJA1, ↓LDLR, ↓MCM4, ↓OAT, ↓PCNA, ↓PTTG1
IL1B	Cytokine	Inhibited	-2.102	$8.57x10^{-4}$	↓CYP19A1, ↓FST, ↓GJA1, ↓INHBA, ↑IRF7, ↓LDHA, ↓LDLR, ↑MYH11, ↑PSMB10, ↓TNFAIP6, ↓VCAN

* Threshold score of ±2.00 was used to identify activated and inhibited upstream regulators.

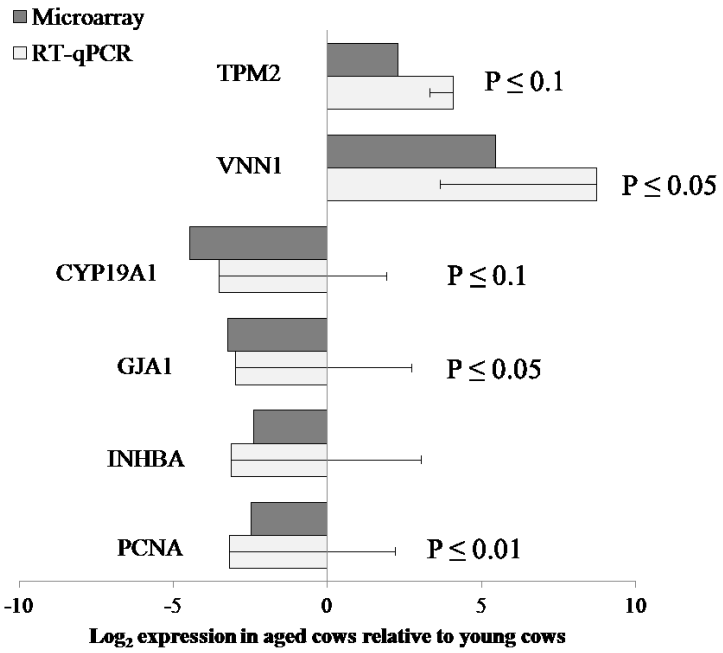

Figure 4.5 Expression of granulosa cell transcripts (log_2 of fold-change) in aged cows relative to young cows. Solid bars (dark gray) represent expression of transcripts by microarray analysis (granulosa cell samples: n=3 per age group). Open bars with standard error (capped line) represent the expression of the same transcripts obtained by RT-qPCR analysis (granulosa cell samples: n=4 per age group). *P*-values denote the differences between aged and young cows in RT-qPCR analyses.

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4.4 Discussion

This is the first study exploring the effect of maternal age at the molecular level using bovine granulosa cells at the time of selection of dominant follicles. Down-regulated transcripts in the granulosa cells of aged cows were related to the lipid and cholesterol metabolism, steroidogenesis, cellular proliferation, replication and DNA repair, intercellular communication, control of gonadotropins, cytoskeleton and extracellular matrix remodelling. Transcripts related to oxidative stress were up-regulated in aged cows. The influence of upstream regulators such as FSH, estrogen, EGF, SREBF-1 and SREBF-2 on granulosa cell function were decreased while the actions of the tumor protein p53 and mitogen-activated protein kinase 14 on granulosa cell were activated in aged cows. Finally, this study suggested that transcriptional profile of the dominant follicle at the time of follicle selection in aged cows differed from healthy follicle and akin to subordinate or atretic follicle. To further this notion, a comparison of dominant follicles of this study with healthy dominant follicle from previous studies has been shown in Table 4.5.

Decreased cellular proliferation and capacity to repair damaged DNA in granulosa cells of aged cows corroborated well with a previous study in which an age-associated decline in DNA repair of human lymphocytes and fibroblast was reported (Goukassian *et al.*, 2000). In the present study, the age-associated reduction in mRNA for PCNA may be attributed to inactivation of upstream molecules such as FSH (Yu *et al.*, 2005), EGF (Lin *et al.*, 2011), activins (El-Hefnawy & Zeleznik, 2001) and activation of TP53 (Xu & Morris, 1999), as shown in Table 4.4. Lower expression of PCNA has been associated with decreased proliferation of granulosa cells in aged cows and rats compared to young (Oktay *et al.*, 1995; Tanno *et al.*, 1996). Previous studies have documented that the ovulatory follicle of 2-wave cycles is smaller in 13-14 year old cows compared to their 1-4 year daughters but such effect was not observed for the first wave

dominant follicles (Malhi *et al.*, 2005). Despite lower PCNA expression detected in the granulosa of aged vs. young cows in the present study, dominant follicle diameter and growth profiles did not differ ($P > 0.05$) between age groups (Fig. 4.1). Perhaps the influence of lower levels of PCNA is expressed in functional rather than physical characteristics of the dominant follicle at the time of selection.

In bovine granulosa cells, Connexin 43 protein encoded by GJA1, is the main constituent of gap junctions, and is needed for cell survival, signal transduction and steroidogenesis (Johnson *et al.*, 1999; Kidder & Mhawi, 2002; Gershon *et al.*, 2008). It has been shown in previous studies that estrogen (Wiesen & Midgley, 1994), gonadotropins (Burghardt & Matheson, 1982; Kalma *et al.*, 2004) and cAMP (Furger *et al.*, 1996) regulate the expression of GJA1; therefore, the potential age-associated decline in intracellular communication (GJA1) in the present study may be attributed to the suppressed response of granulosa cells of aged cows to upstream regulators like FSH and estrogen (Table 4.4). Similar age associated decline in the capacity of synthesizing gap junctions has been reported in human osteoclasts (Genetos *et al.*, 2012). The *Gja1*-null mouse model showed reduced PCNA expression and DNA synthesis (Gittens *et al.*, 2005), thus underscoring the need of gap junctions for cell growth. Likewise, decreased mRNA expression of GJA1 in granulosa cells from aged cows may be a reason for the observed age-associated reduction in PCNA.

Previous studies demonstrated a direct association between the mRNA levels for aromatase (CYP19A1) and estrogen biosynthesis (Simpson *et al.*, 1994; Sisco & Pfeffer, 2007). In the present study, aged cows showed decreased transcripts of CYP19A1 in granulosa cells; however, intrafollicular concentrations of estradiol 17β were not different (albeit, numerically lower from those in young animals (Table 4.2). Older cows have high plasma levels of FSH

(Malhi et al 2005) and FSH along with insulin and insulin like growth factor 1 (IGF-1) has been shown to control the CYP19A1 expression in bovine granulosa cells (Silva & Price, 2002). In this study, age-associated lower expression of CYP19A1 in aged cows is perplexing, however, > 4-fold decrease in CYP19A1 transcripts detected by microarray analysis and confirmed by quantitative PCR, may be due to lower responsiveness of granulosa cells to FSH (upstream regulator, Table 4.4). Lower transcripts of CYP19A1 in part with decreased estrogen synthesis have also been associated with reduced proliferation or cell survivability, and inadequate development of gap junctions in granulosa cells (Burghardt & Anderson, 1981; Segaloff *et al.*, 1990; Evans *et al.*, 2004; Valdez *et al.*, 2005). Therefore, it is hypothesized that observed lower expression of CYP19A1, GJA1 and PCNA mRNA in granulosa cells of aged cows may contribute, in part, towards suboptimal follicular function and lower oocyte competence.

In this study, a potential cause of 1.5 times reduction in intrafollicular progesterone levels in the dominant follicle (Table 4.2) from aged cows may be restricted cholesterol synthesis or lipid processing because of inhibition of granulosa cell responsiveness to transcription factors SREBF1 and SREBF2 (Table 4.4). Both SREBF1/2 have been shown to regulate all of the cholesterol synthesis pathways genes and repression of both or one of these resulted in lower mRNA levels of LDLR, SCD and HMGCS1, CYP51A1 and others cholesterol synthesis genes (Sakakura *et al.*, 2001). In this context, lower expression of cholesterol synthesis genes (Supplementary Table 10.1) in granulosa cells of aged cows suggested decreased processing of cholesterol. The lower mRNA of SCD in granulosa cells of aged cows also suggested increased amount of free cholesterol and activation of LXR pathway (Fig. 4.3B) and were consistent with the findings of previous study (Paton & Ntambi, 2010). Not surprisingly, increased expression of *CD36* (Supplementary Table 10.1) in granulosa cells of aged vs. young cows suggested

greater dependency of granulosa cells on high density lipoprotein (HDL) mediated uptake for cholesterol rather than on low density lipoprotein (LDL) mediated cholesterol uptake and on de novo cholesterol synthesis. Our interpretation is that maternal aging results in suboptimal uptake and utilization of cholesterol for steroidogenesis in granulosa cells.

The cytoskeleton and extracellular matrix (ECM) serve important roles during folliculogenesis (Grieshaber *et al.*, 2003; Woodruff & Shea, 2007). Levels of TNFAIP6 and VCAN transcripts were decreased -3.5 and -2.0 folds, respectively in granulosa cells from aged cows. TNFAIP6 and VCAN stabilize the ECM (Carrette *et al.*, 2001; Rodgers *et al.*, 2003), regulate cell migration, and are involved in mucification (Fulop *et al.*, 2003), antrum formation, and remodeling of cell junctions (Rodgers & Irving-Rodgers, 2010). In cumulus cells, higher expression of *VCAN* has been proposed as a biomarker for the fertilizing potential of an oocyte, pregnancy and live birth (Gebhardt *et al.*, 2011). It is also noteworthy that higher expression of *TPM2* recorded in the granulosa cell from older cows is indicative of less differentiated granulosa cells (Baum *et al.*, 1990). Therefore, decreased expressions of TNFAIP6, VCAN and Tubulins, along with increased expression of TPM2, are consistent with lower ECM stability, less differentiation, and reduced steroidogenesis in granulosa cells of aged vs. young cows.

Previously, age-associated higher plasma FSH levels were reported in aged cows (Malhi *et al.*, 2005); basal FSH concentrations were high during the entire interovulatory interval; however, the association between follicular wave dynamics and plasma FSH concentrations remained unaltered as compared to younger animals. In the current study, decreased mRNA levels of inhibin B (alpha and beta subunits) and follistatin in granulosa cells of aged cows suggested the basis of high plasma FSH levels in aged cows. Granulosa cells of bovine dominant follicles contained more follistatin than corresponding subordinate follicles and the amount of

follistatin was associated with the period of functional dominance (Singh & Adams, 1998). Both inhibins and follistatin (FST) belong to transforming growth factor (TGF) family and regulate the release of FSH from anterior pituitary gland (Carroll *et al.*, 1989). As a consequence of altered production of these factors by the granulosa cell of aged cows, there is potentially less negative feedback on anterior pituitary to contain FSH secretion. In support, high plasma FSH levels in aged women have been associated with decreased mRNA levels of inhibins and follistatin (Welt *et al.*, 1999). In this study, expression of INHBA from microarray analysis could not be confirmed by RT-qPCR due to greater biological differences and limited samples (n=4 per group), however, RT-qPCR analysis showed down-regulation in the expression of INHBA similar to microarray analysis (Fig. 4.5). In summary, a possible explanation of decreased FSH regulation during the maternal aging may be the decreased mRNA synthesis of inhibins and follistatin by granulosa cells of the dominant follicle in aged cows.

Oxidative stress has been described as a major player in age-associated changes in female reproduction (Agarwal *et al.*, 2005) and previous studies showed that oxidative/inflammatory stress resulted in increased expression of VNN1, VNN2 and GPX3 (Maras *et al.*, 1999; Berruyer *et al.*, 2004; Bonnet *et al.*, 2006; Zhang *et al.*, 2011). Both VNN1 and VNN2 have been described as epithelial enzymes and were shown to regulate cysteamine and glutathione metabolism (Maras *et al.*, 1999). It is worth mentioning that VNN1 and VNN 2 were the two top up-regulated transcripts detected in the microarray study showing an increase of 5.5 and 3.8 folds, respectively, in the granulosa cells of old cows. Similarly, transcription of GPX3, that encodes glutathione peroxidase 3, was 2.3 fold higher in old cows. Glutathione peroxidase catalyzes the process of reduction of hydrogen (H_2O_2) and lipid peroxides into reduced glutathione (GSH). High levels of GPX3 have been suggested in protecting follicle from

oxidative stress induced atresia by regulating free hydrogen peroxides (Bonnet *et al.*, 2006). In an earlier study, activation of tumour protein TP53 and increased expression of GPX3 have been suggested as part of post-stress survival mechanisms (Horn & Vousden, 2007). Consistent with this finding, transcription of both (TP53 and GPX3) was up-regulated in granulosa cells of aged cow in response to oxidative stress (Table 4.4 and Fig. 4.4). Once activated, TP53 has been shown to regulate cell cycle (Xu & Morris, 1999). Depending on the severity of the DNA damage, TP53 either activates cell death mechanism or represses cell cycle genes (PCNA, PTTG1 etc., supplementary Table 10.1) to check DNA damage (Xu & Morris, 1999). Interestingly, both outcomes i.e., the higher rate of cell death and decreased granulosa cell proliferation are present in the dominant follicle of aged cows (Fig.4.3). Besides TP53, activation of MAPK14 *(p38)* also indicated DNA damage (Table 4.4) in granulosa cells of aged cows at the time of dominant follicle selection. The potential reason of MAPK14 activation in aged cows may be due to decreased ratio of reduced to oxidized glutathione (GSH: GSSH) (Limon-Pacheco *et al.*, 2007). In agreement, oxidative stress induced MAPK14 activation has previously been reported in granulosa cells of aged cows (Ito *et al.*, 2008). Thus, the microarray analysis of the granulosa cells at the time of selection of the dominant follicle indicated oxidative stress in aged cows.

Table 4.5 Bovine genes regulated during dominant follicle selection and maternal aging. Granulosa cell genes previously associated with follicular dominance (dominant vs subordinate follicles) were consistently under-expressed in the dominant follicle of aged cows compared to those from young cows. Nagative values in parentheses indicate fold change (down-regulation) in aged cows recorded in the present study.

Gene	Function	Expression in granulosa cells		Reference
		Dominant follicle (aged vs. young cows)*	Dominant vs. subordinate follicle**	
CYP19A1	Estradiol synthesis, cell differentiation	down-regulated (-4.5)	up-regulated	Mihm et.al 2008 Evans et al 2004 Fayad et al. 2004 Sisco et al. 2003
INHBA	Cell differentiation and inhibin synthesis	down-regulated (-2.4)	down-regulated up-regulated up-regulated	Mihm et.al 2008 Fayad et al. 2004 Sisco et al. 2003
GJA1 or Cx43	Intercellular communication and transportation	down-regulated (-3.2)	up-regulated	Fayad et al. 2004
RGN	Ca^{++} homeostasis, Senescence marker protein	down-regulated (-2.2)	up-regulated	Fayad et al. 2004
LRP8 or ApoER2	Signal transduction, lipid metabolism	down-regulated (-3.2)	up-regulated	Fayad et al 2004 Liu et al. 2009 Sisco et al. 2003
CSPG2 or VCAN	Cell adhesion, survival, proliferation, migration and extra cellular matrix assembly	down-regulated (-2.0)	up-regulated	Fayad et al. 2004
TNFA1P6	Extracellular matrix stability, cell migration and cell matrix interaction	down-regulated (-3.5)	up-regulated	Fayad et al. 2004
PTTG1	Cell Cycle, DNA repair	down regulated (-2.0)	up-regulated	Liu et al. 2009
STRA6	Lipid metabolism, Vitamin A metabolism	down-regulated (-2.1)	up-regulated	Mihm et al. 2008
SCD	Lipid synthesis (desaturation of stearic acid)	down-regulated (-3.2)	up-regulated	Mihm et al. 2008

* Data from present study

** Results based on previous studies listed under Reference column.

In conclusion, transcriptome analysis of granulosa cells of the dominant follicle, near the time of selection, indicated alterations in the expression of specific genes in aged cows. Transcripts that were most affected by maternal age were those associated with cellular proliferation, lipid or cholesterol metabolism, steroidogenesis, intracellular communication, cytoskeleton and extracellular matrix, and gonadotropin regulation. Genes associated with dominance were under-expressed in the granulosa cells of aged cows at the time of dominant follicle selection. If associated with changes in the respective protein levels, alterations in gene expression may explain the inability of the follicles from aged cows to properly differentiate and progress towards ovulation. Cellular and molecular mechanisms uncovered in this study extend and validate the bovine model of human reproductive aging. By designing clinical interventions to control oxidative stress, to modulate lipid metabolism and steroidogensis, and to enhance gonadotropin responsiveness, it may be possible to compensate for the deleterious effects of advancing maternal age on fertility in both humans and animals.

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5 CHAPTER 5: MATERNAL AGE RELATED CHANGES IN TRANSCRIPTOME OF BOVINE GRANULOSA CELLS OF PREOVULATORY FOLLICLE 24 H AFTER LH TREATMENT

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Roles and Contributions of Co-authors: Fernanda Dias helped in tissue collection, sample processing and setting up RT-qPCR. Marc-Andre helped in data analysis and critically reviewed the manuscript. Gregg Adams helped in tissue collection, study design and critically reviewed the manuscript and Jaswant Singh supervised, helped in study design, data analysis and critically reviewed the manuscript.

Relationship of this study to the dissertation

In the earlier study of the dissertation, granulosa cells of the dominant follicle at the time of selection were analyzed to determine age-associated changes in the transcriptome. In this study, granulosa cells of the preovulatory follicle 24 h after LH treatment were analyzed to see how maternal age alters the function of the ovulatory follicle and leads to ovulatory dysfunction. Delayed and fewer ovulations have been reported in the bovine model of reproductive aging. This study explores the pathways and molecular functions that are compromised due to advanced maternal age in cows and may result in delayed ovulation.

5.1 Introduction

Leutinizing hormone (LH) surge initiates a cascade of events in the wall of a preovulatory follicle that leads to marked changes in differentiation, inflammation, luteinization and steroidogenic pattern (Richards *et al.*, 1998; Murphy *et al.*, 2001; Richards *et al.*, 2002; Richards *et al.*, 2002). Collectively, these events are associated with the timing of ovulation, oocyte maturation, and fertility. Breeding management in cows (e.g., fixed-time artificial insemination, superovulation and embryo transfer) and infertility treatments in women (e.g., intrauterine insemination and controlled ovarian hyperstimulation) rely on optimal and timely response of granulosa cells to such an ovulatory stimulus. In aged women and cows, follicular and luteal dynamics indicate delayed occurrence of ovulation in response to the preovulatory surge in gonadotropins. Compared to their young daughters, the proportion of the ovulations in old cows was significantly lower between 24 and 48 h after LH administration (94 vs. 84%, respectively), and higher between 48 and 72 h after LH administration (4% vs. 13%, respectively) (Malhi *et al.*, 2008). The inability to produce a mature oocyte or failure to ovulate has been reported in ~25% of women undergoing treatment for infertility (McClure N, 1997). In aged women, ovulation occurred later in the menstrual cycle (monitored by daily ovarian ultrasonography) due to a prolonged follicular phase (Fitzgerald *et al.*, 1994). In menstrual cycles in which conception was achieved, ovulation occurred within 24 h after the LH surge, whereas the interval to ovulation was extended up to 48 h after LH in non-conception cycles (Zegers-Hochschild *et al.*, 1984). The percentage of immature oocytes obtained after ovarian hyperstimulation during IVF treatment was also higher in women ≥ 40 years of age [22]; even though the total number of retrieved oocytes and immature oocytes were lower in aged women ≥ 40 as compared to women < 40 years of age [22]. In a bovine study, 71% of oocytes collected

from aged cows remained unfertilized or uncleaved after IVF compared to 38% of oocytes collected from their young daughters (Malhi *et al.*, 2007). An age related delay in ovulation has been associated with a delay in the LH surge in cows (Malhi *et al.*, 2008) and a decrease in the magnitude of the LH surge in rats (Matt *et al.*, 1998; LaPolt & Lu, 2001), both of which suggest altered function or sensitivity of the hypothalamo-pituitary axis. Collectively, these findings suggest that maternal aging is associated with a delay in the preovulatory LH surge, delayed ovulation, and decreased oocyte competence.

Progesterone is rapidly synthesized by follicular cells after the LH surge in women (Veldhuis *et al.*, 1983) and cows (Fortune & Quirk, 1988). In lactating dairy cows, high rate of early embryonic mortality has been associated with lower concentrations of estradiol and subsequent lower concentrations of progesterone from the steroidogenic cells of the preovulatory follicle (Inskeep, 2004). Intrafollicular concentrations of progesterone were significantly lower in lactating cows compared to non-lactating heifers and were related to altered metabolic activity (lipid and fatty acid metabolism) of the follicular cells and to fertility (Bender *et al.*, 2010; Walsh *et al.*, 2012). Perhaps such a scenario exists in aged cows since luteal phase plasma progesterone concentrations were lower and oocyte competence was decreased in aged cows compared with their young daughters (Malhi *et al.*, 2005; Malhi *et al.*, 2007).

Based on the studies cited above and others (Erickson *et al.*, 1976; Klein & Sauer, 2001), it is evident that maternal aging in animals and humans is associated with decline in fertility. For ethical and practical reasons, human ovarian tissues (oocytes, follicles, corpus luteum) cannot be obtained for research and hypothesis testing. To overcome these limitations, our research group has validated the use of bovine model (Adams & Pierson, 1995; Adams *et al.*, 2012) to study the maternal aging (Malhi *et al.*, 2005; Malhi *et al.*, 2006; Malhi *et al.*, 2007;

Malhi *et al.*, 2008). Elevated circulating concentrations of FSH and reduced ovarian reserve in 13-15 year old cows (Malhi *et al.*, 2005) were comparable to that reported in women approaching menopause. Further, maternal aging in cattle was associated with 1) a delay in the preovulatory LH surge (Malhi *et al.*, 2006), 2) a decline in follicular and ovulatory response after superstimulatory treatment (Malhi *et al.*, 2008) and 3) markedly decrease in oocyte competence (Malhi *et al.*, 2007). In a recent report, granulosa cells of the dominant follicle at the time of follicle selection in aged cows were predicted to be associated with inefficient gonadotropin regulation, lipid processing and steroid synthesis, decreased cell proliferation and higher oxidative stress response (Khan *et al.*, 2012). These findings suggest that the structure and function of granulosa cells of ovarian follicles are compromised with increased maternal age, and may lead to diminished capacity of the granulosa cells to perform post-LH surge-related tasks required for ovulation and CL development. Transcriptome analyses of granulosa cells during preovulatory maturation (Martoriati & Gerard, 2003; Tetsuka *et al.*, 2010) and luteinization (Wu & Wiltbank, 2002; Gilbert *et al.*, 2011) highlight the role of granulosa cells during final stages of oocyte maturation and attainment of developmental competence. However, there is limited information available about age-associated changes in granulosa cells (Ito *et al.*, 2008; Hurwitz *et al.*, 2010; Ito *et al.*, 2010). The objective of the study was to compare changes in gene expression of granulosa cells of preovulatory follicles (collected 24 h after treatment with LH) from aged cows vs. young cows. We hypothesized that delayed ovulation in aged cows is associated with 1) altered gene expression of granulosa cells of the preovulatory follicle and 2) decreased production of progesterone by the granulosa cells of the preovulatory follicle.

5.2 Materials and methods

5.2.1 Animals

Hereford-cross beef cattle (n=12) were maintained together in a single corral at the University of Saskatchewan Goodale Farm. They were born on this farm so their birth records and calving history records were available. The cows had not been pregnant nor lactated in the previous two years.Animals were divided in aged cows (17 ± 2.5 years, mean ± SEM; n=6) and young cows (9 ± 0.6 years; n=6). Five out of six young cows were daughters of the aged cows. One aged cow did not had a daughter, so was matched with a younger cow from the herd whose age was similar to the duaghters. The experimental protocol was approved by the University of Saskatchewan Committee on Animal Care and procedures were conducted in compliance with the guidelines of the Canadian Council on Animal Care.

5.2.2 Ultrasonography, hormonal treatments and follicular dynamics

For the purpose of granulosa cell collection (next section), all cows were given a luteolytic dose of prostaglandin ($PGF_{2\alpha}$; Lutalyse, 25 mg i.m., Pfizer Animal Health, Kirkland, QC, Canada) to synchronize ovulation. The ovaries were examined daily from the day of PGF2α to the day of tissue collection by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SD 900, Tokyo, Japan) to record the size and location of corpus luteum and all follicles >3mm. Ovulation was defined as the day on which a large follicle disappeared on ultrasonographic examination (Malhi *et al.*, 2005). The interval to ovulation after $PGF_{2\alpha}$ administration (mean hours ± SEM) was compared between the aged and young cows (n=6 per age group). The day of ovulation was taken as the day of emergence of the first follicular wave (Day 0) (Singh *et al.*, 1997; Malhi *et al.*, 2005). The dominant follicle was identified

retrospectively based on the ultrasonographic record as the largest follicle of the wave, and the next largest follicle was defined as largest subordinate follicle (Ginther *et al.*, 1989; Jaiswal *et al.*, 2004). On Day 4.5 and 5, cows were given $PGF_{2\alpha}$, i.m. to induce luteolysis. On Day 6, cows were given LH (Lutropin, 25 mg i.m., Bioniche Animal Health, Belleville, ON, Canada) and on Day 7, granulosa cells were harvested. Follicular data (diameters of dominant and largest subordinate follicles, growth rates) were compared between the aged and young cows (n = 6 records per group).

5.2.3 Tissue collection for micorarrays and RT-qPCR

Granulosa cells for microarray and RT-qPCR analyses were obtained by ultrasound-guided follicle aspiration of preovulatory dominant follicle 24 hr after LH injection (animal treatments decribed in previous section). Granulosa cells from 3 of the 5 mother-daughter pairs were used for microarray experiments (n=3 per age group). Follicle aspirates from remaining 6 animals were used for real-time quantitative PCR (RT-qPCR) analysis. In addiditon, antral granulosa cells from one aged cow and two young cows from the microarray group were obtained by unilateral ovariectomy at a later time. Therefore, RT-qPCR analyses were perfomed using mRNA samples from 4 aged cows and 5 young cows.

Transvaginal ultrasound-guided follicle aspiration was done using a 5 MHz convex-array transducer (Aloka SSD 900, Tokyo, Japan) equipped with a disposable needle (catalog # 305833, 18 ga x 1.5”; BD Medical, Mississauga, ON, Canada) connected to a 10 ml syringe by silicone tubing (115 cm long, 1.14 mm internal diameter, Cole-Palmer, Montreal, QC, Canada), as described (Berfelt *et al.*, 1994). Caudal epidural anesthesia was induced, and perineum was disinfected as described above. After introducing the transducer into the vaginal fornix, the ovary was positioned against transducer face by transrectal manipulation, and the dominant and largest

subordinate follicles were punctured and follicular contents aspirated. The cumulus-oocyte-complex (COC) was identified under a stereomicroscope and separated from the aspirate. The follicular fluid was centrifuged at 700g for 15 minutes to harvest the pellet of antral granulosa cells. Follicular fluid was stored at -80 °C. Granulosa cells were either snap frozen in liquid nitrogen and stored at -80 °C or suspended in 300-700 µl of RNA stabilization and protection solution (RNA*later*, catalog # AM7020; Life Technologies, Burlington, ON, Canada) for 12 h at 4 °C before storing at -80 °C for later microarray analysis.

Unilateral ovariectomies were performed by colpotomy (Singh *et al.*, 1998) as described. Briefly, caudal epidural anesthesia was induced with 2% lidocaine HCl (catalog # 1LID009P; Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). An incision was made in the dorso-lateral aspect of the vaginal fornix. The peritoneum was ruptured manually and local anesthesia was applied to ovarian pedicle using lidocaine-soaked gauze. An ecraseur (Jorgensen Labs, catalog # J0037E, Loveland, Colorado, USA) was used to cut the ovarian attachments by slowly tightened a chain around the ovarian pedicle. The cows were treated with procaine penicillin G (Pen G injection; 21000 IU/Kg, i.m., Citadel Animal Health, Edmonton, AB, Canada) daily for four days after ovariectomy. The ovary was kept on ice and transported to the laboratory within 5 minutes after collection. The size of the dominant follicle and the largest subordinate follicle (if located ipsilateral to the dominant follicle) were confirmed by measuring the diameter. The goal was to collect the antral granulosa cells (those located close to the antrum) of the dominant follicle (in contrast to the mural granulosa cells that are located close to basement membrane). The follicular contents were removed using a 20 gauge needle attached with 3 ml syringe. The needle was left in place and the collapsed follicle was flushed 3-times with Delbecco's phosphate buffer saline (DPBS, catalog # 21600-010; Life Technologies,

Burlington, ON, Canada). The cumulus-oocyte-complex (COC) was identified, separated from the aspirate and discarded. Contents of the first syringe was centrifuged at 700g for 5 minutes at 4 °C to harvest follicular fluid and the cell pellet was mixed with granulosa cells from DPBS contents, centrifuged again at 700g for 15 minutes at 4 °C. Follicular fluid and granulosa cells were processed and stored similar to those obtained by ultrasound-guided follicle aspirations.

5.2.4 Follicular fluid analysis for estradiol and progesterone

Follicular fluid from aged (n= 4 dominantfollicles) and young (n= 5 dominant follicles) cows were subdived into three age groups, i.e., aged (n=2 dominant follicles), middle aged (n= 3 dominant follicles) and young (n=4 dominant follicles) due to the variability in age of the cows. Follicular fluid was analyzed for estradiol 17β and progesterone concentrations using radioimmunoassay (RIA) as described (Singh *et al.*, 1998). Briefly, standards and the sample dilutions were prepared by using charcoal-extracted pooled bovine follicular fluid from abattoir-collected ovaries. For estradiol 17β assay, follicular fluid samples were diluted at 1:100 or 1:500 v:v with charcoal-extracted follicular fluid and a commerical RIA kit (catelog # KE2D1, Coat-A-Count, Siemens Health Care Diagnostics Inc., Malvern, PA, USA) with sensitivity of 5-500 pg/ml was used. For progesterone assay, samples were diluted 1:40 v:v and a commercial RIA kit (catalog # TKOP1, Coat-A-Count, Siemens Health Care Diagnostics Inc.) with sensitivity of 0.1-40 ng/ml was used. All samples were processed as duplicates in a single run. Intra-assay coefficients of variations for estradiol and progesterone assays were 11% and 2.5%, respectively.

5.2.5 Granulosa cell RNA extraction, amplification and labeling

RNA extraction, amplification and labeling of granulosa cells was done as previously described (Dias *et al.*, 2013). Briefly, total RNA of granulosa cells was extracted (RNA Isolation Kit # KIT0204; Life Technologies, Burlington, ON, Canada) and eluted in 15 µl of the buffer as

prescribed in the manufacturer's protocol. Quality and quantity of RNA were determined (2100-Bioanalyzer; Agilent Technologies, Santa Clara, CA, USA). For microarray analysis, 5 ng of total RNA was linearly amplified (RNA amplification Kit # KIT0525; Life Technologies, Burlington, ON, Canada) and 2 μg of antisense-RNA was labeled with Cy3 and Cy5 florescent dyes (ULS florescent labeling kit # EA-021; Keratech Biotechnology, Amsterdam, The Netherlands) according to the manufacturers' protocols. Labeling efficiency for both dyes was measured by spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the non-reacted residual labeling dye was filtered out without DNAse treatment (RNA isolation kit # KIT0204; Life Technologies, Burlington, ON, Canada).

5.2.6 Microarrays

For microarray analysis, three biological replicates (each replicate consisted of one aged cow and her daughter) were compared directly on three separate microarrays. The bovine oligo-array slide was custom-designed (EmbryoGENE EMBV3, Design ID: 028298, GEO accession # GPL13226; Agilent Technologies) and consisted four arrays of 37,351 genes or isoforms (excluding the control spots) in a 4x44K format (Robert *et al.*, 2011). Briefly, in each of three biological replicates, 825 ng of Cy3-labeled antisense-RNA from an aged cow and 825 ng of Cy5-labeled antisense-RNA from her daughter (young cow) were mixed and hybridized on an array. To control for non-biological variation, each of the three biological replicates had a technical replicate; i.e. Cy3 and Cy5 dyes were reversed in a biological replicate (dye swap) and hybridized to a separate array (Robert *et al.*, 2011). In total, six hybridizations were performed that included three biological and three technical replicates. Microarray slides were incubated at 65 ºC for 17 h in a rotating oven at 10 rpm. After washing and drying the slides as previously described (Dias *et al.*, 2013), slides were scanned by a Power Scanner (Tecan Group Ltd.

Mannedorf, Switzerland) for image acquisition. Images were quantitated for signal intensity using Array-Pro software (Media Cybernetics Inc., Rockville, MD, USA).

Signal intensity data were processed and statistically analyzed as described (Dias *et al.*, 2013). Briefly, the MIAME-compliant platform of EmbryoGENE Laboratory Information Management System and the microarray analysis (http://elma.embryogene.ca) was used to assess linearity, specificity, and variability of hybridization (http://elma.embryogene.ca). Briefly, the signal intensity of the background of a target was subtracted from the median signal intensity of the foreground of the target. The median pixel value for each target was transformed to the $\log_2$ and subjected to "within array" and "between-array" normalization using quantile method and lowess nonparametric regression method. Finally, a list of differentially expressed genes was obtained by running simple limma algorithm (Michal Blazejczyk, 2007). A gene was considered to be differentially expressed when a $\geq$ 2-fold change in its expression at *P*-value of $\leq$ 0.05, was detected. Benjimeni-Hocheberg method with a fold change of $\geq$ 2 and *P*-value of $\leq$ 0.05 was used to determine true positive genes. Data were deposited in NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) with a GEOseries accession number of (This will be acquired once the manuscript is ready for submission)

5.2.7 Functional analyses

To understand the biological context of the differentially expressed genes ($\geq$ 2-fold change; $P \leq 0.05$) in granulosa cells of aged cows vs. young cows, Pathway analysis software (Ingenuity Pathway Analysis; www.ingenuity.com) was used. Genes that matched the Ingenuity knowledge-base were processed for functional annotation, pathways, networks and upstream regulator analyses.

5.2.8 Real time quantitative polymerase chain reaction (RT-qPCR)

Real-time qPCR analysis of six genes (VNN1, GADD45B, TNFAIP6, SERPINE2, NAR5A2 and RGS2) was done on samples from aged (n=4) and young cows (n=5) to validate the microarray analysis. Briefly, 50 ng of total RNA was converted into complimentary-DNA (cDNA) using q-Script (catalog # 95047-100; Quanta Biosciences Inc., Gaithersburg, MD, USA). Primer pairs of each gene were designed (Primer 3; http://frodo.wi.mit.edu/primer3/), analyzed for compatibility (OligoAnalyzer 3.1; Integrated DNA Technologies) and dissociation curve and amplification efficiency (MxPro software; www.genomics.agilent.com; Table 5.1), and confirmed by Basic Local Alignment Search Tool (BLAST) against the NCBI database (www.blast.ncbi.nlm.nih.gov/). For each gene, the size of PCR product was determined (1% agarose gel-electrophoresis), and PCR product gel-bands were eluted from gel (QIAquick gel extraction kit # 28704; Qiagen) and sequenced (ABI 3730 XL DNA analyzer; Applied Biosystems) to determine the quality (Gap 4.4 software; Staden-Package; http://www2.mrc-lmb.cam.ac.uk/) and specificity (BLAST). Standard curve ranging from 10^{-2} to 10^{-9} ng/µl was prepared from the purified and sequenced PCR product of each gene and used to calculate the relative amounts of cDNA of a gene in the samples from aged and young cows.

RT-qPCR were perfomed as previously described (Dias *et al.*, 2013). Briefly, a separate PCR was set up for each gene, which consisted a standard curve, no template control (NTC) and cDNA of granulosa cells. Single reaction was run for all granulosa cell samples while multiple reactions were set up for the standard curve and NTC. Total volume of each PCR reaction was 25 µl and composed of 2 µl of cDNA of each sample, 12.5 µl of SYBR green master mix II (catalog # 600828; Agilent Technologies, Burlington, ON, Canada), 1875 nM of each forward and reverse primer, 0.375 µl of reference dye and 6 µl of nuclease-free water. For the analysis of

RT-qPCR, the relative amount of each gene was normalized using the geometric mean of four reference genes (UBE2D2, EIF2B2, GAPDH and SF3A1) (Khan *et al.*, 2013). The normalized quantity of each gene was compared statistically between aged vs. young cows using REST software (Qiagen 2009) (Pfaffl *et al.*, 2002).

5.2.9 Statistical Analyses for follicular and hormonal datasets

Daily follicular diameters and growth rates were analyzed using repeated measures PROC MIXED procedure (SAS version 9.2; SAS Institute Inc., Cary, USA). Dominant follicle data were compared for the main effects (age and day of wave) and their interactions (age*day). Dominant and subordinate within each age group were compared for follicle type, day and their interactions. Intrafollicular estradiol 17β and progesterone concentrations of dominant follicles were compared by one-way analysis of variance.

Table 5.1 List of primers used for RT-qPCR analysis to validate microarray results. The genes and details of primers along with expected amplicon size and obtained PCR reaction efficiency are provided.

Gene	Accession Number	Oligo	Primer pair Sequence (5' to 3')	Amplicon size (bp)	Efficiency (%)
Target Genes					
NR5A2	NM_001206816.1	Forward	AACAGAAAAAGAACACGGAAG	157	107.3
		Reverse	CTACTGGGGAAGATTTGAAGCAC		
RGS2	NM_001075596.1	Forward	AAATGTGGTGTGTCTCAAGCTG	232	102.4
		Reverse	AGGAACTTCAGTACCCTTGCAC		
TNFAIP6	NM_001007813.2	Forward	AAGGAGTGTGGTGGTGTGTTTA	185	103.0
		Reverse	TCAACATAGTCAGCCAAGCAAG		
GADD45B	NM_001040604.1	Forward	CCAGGACAGTACTTTGGGACTT	150	108.0
		Reverse	ATCTGTAAGCTTCCCCTCTGTC		
VNN1	NM_001024556.2	Forward	TATTCTCTTCCACGATCCTGCT	197	105.3
		Reverse	TTCCACTCCCTGTCATTTTCTT		
SERPINE2	NM_174669.2	Forward	ATCTTGCATTACTTTGGGGGTA	168	117.2
		Reverse	AGACCAGTAGTTGACAGGCACA		
Housekeeping Genes					
EIF2B2	NM_001015593.1	Forward	CATGAGATGGCAGTCAATTTGT	219	97.3
		Reverse	CTTGAACATAGGAGCACAGACG		
GAPDH	NM_001034034.1	Forward	CCAACGTGTCTGTTGTGGATCTGA	275	99.0
		Reverse	GAGCTTGACAAAGTGGTCGTTGAG		
SF3A1	NM_001081510.1	Forward	TGTGTCCCTCTTGCTGAGTTT	194	96.6
		Reverse	ATTCCTGGTTTCACGTCTCCTA		
UBE2D2	NM_001046496.1	Forward	TGGACTCAGAAGTATGCGATGT	242	102.8
		Reverse	CTTCTCTGCTAGGAGGCAATGT		

5.3 Results

5.3.1 Follicular and hormonal dynamics

The interval to ovulation (mean ± SEM) after $PGF_{2\alpha}$ administration was longer (P = 0.02) in aged (115 ± 2.5 h) than young cows (90 ± 6.0 h). Diameters of the preovulatory follicle, 24 h after LH treatment, did not differ between aged and young cows (Fig. 5.1). As expected, the diameter profile of the dominant follicle was greater than that of the largest subordinate in both age groups (Fig. 5.1). Comparison of intrafollicular concentrations of estradiol 17β and progesterone between groups revealed that aged cow had a lower concentration of progesterone ($P < 0.05$) and maintained a high concentration of estradiol concentrations ($P < 0.05$) as compared with middle-aged and young cows (Fig. 5.2).

5.3.2 Differential gene expression

A total of 1340 genes and gene or isoforms were differentially expressed at threshold of fold change ≥2 ($P \leq 0.05$) in granulosa cells of the preovulatory follicles from aged cows vs. their daughters. Expression levels of 722 genes and isoforms down-regulated while 618 genes and isoforms were up-regulated (Fig. 5.3). Top ten up and down-regulated genes showing largest fold change in their expression are shown in Table 5.2. Relevance of these genes to ovarian physiology is provided as Supplemental Table 10.2.

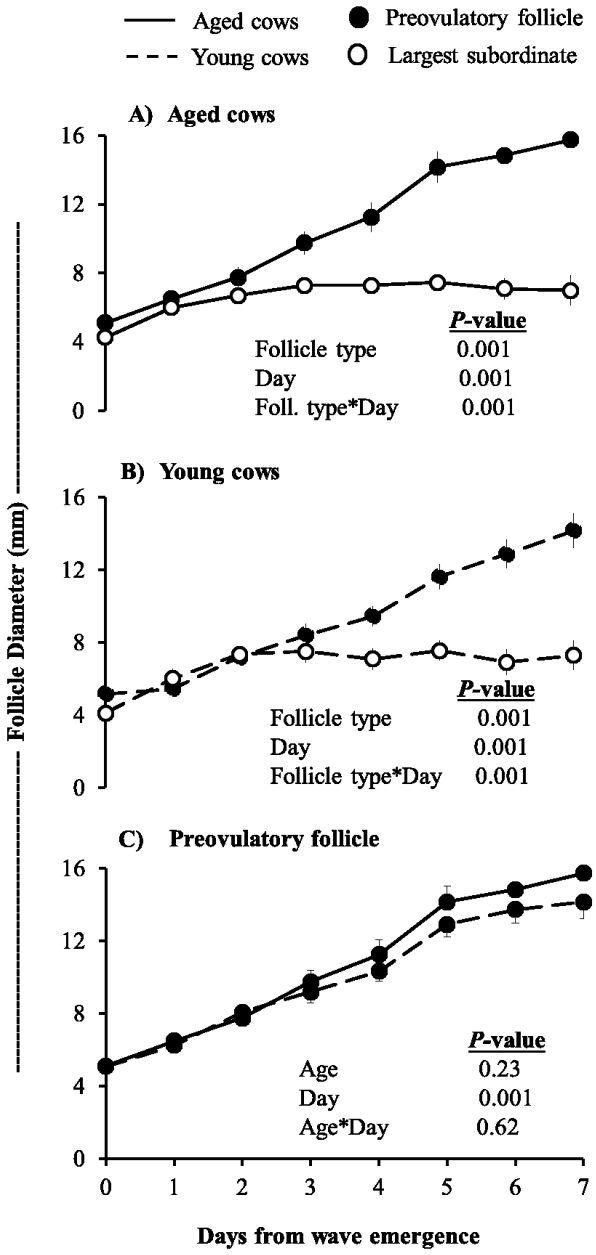

Figure 5.1 Diameter profiles (mean ± SEM) of ovarian follicles of aged (n=6) and young cows (n=6). A) Preovulatory follicle vs. largest subordinate follicle from aged cows. B) Preovulatory follicle vs. largest subordinate follicle from young cows. C) Preovulatory follicle of aged cows vs. young cows. Diameter and of ovarian follicles are aligned to the day of ovulation (emergence of new follicle wave).

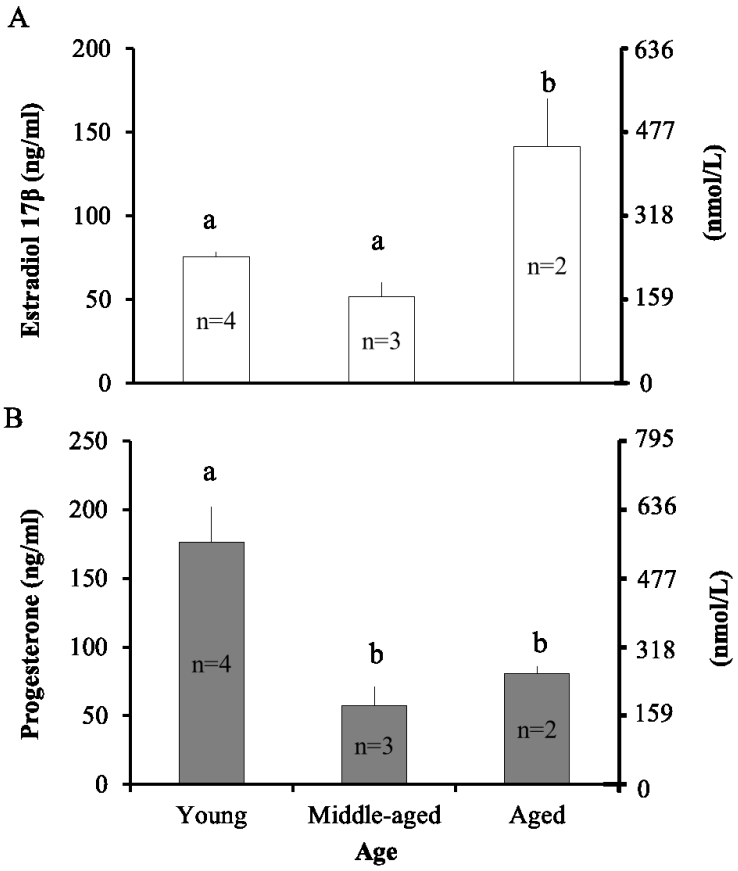

Figure 5.2. Intra-follicle estradiol 17β (A) and progesterone (B) concentrations (Mean ± SEM) in the preovulatory follicle of young (7±0.9 years), middle aged (11±1.0 years) and aged (21±0.5 years) cows. The number inside each bar represents the number of cows in each age group. [a,b] Values with different superscripts are different ($P < 0.05$).

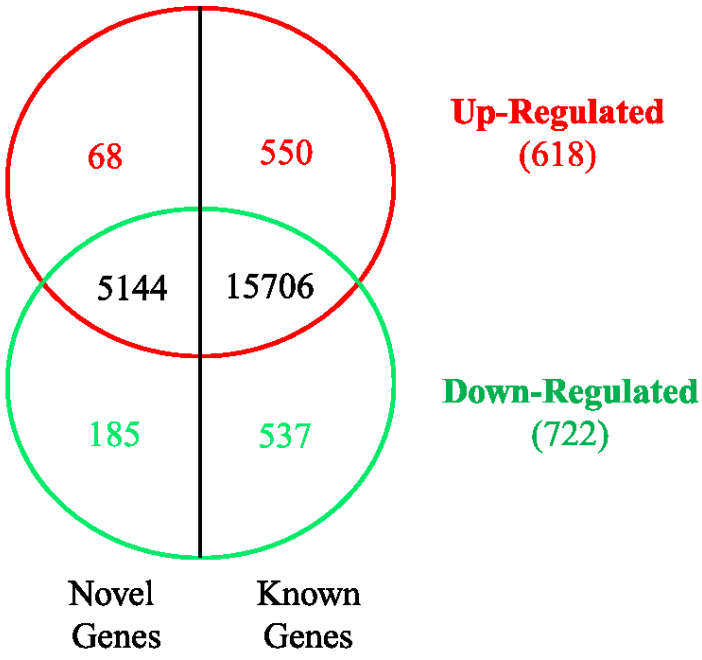

Figure 5.3 Venn diagram representing the number of transcripts up-regulated (red) and down-regulated (green) in granulosa cells of preovulatory follicles of aged vs. young cows. The overlapping region (center) represents transcripts that are common to both aged and young cows. The left half of each circle represents the number of novel genes and right half represents the known genes. A total of 1340 genes or isoforms were differentially expressed in aged cows vs. young cows using a threshold value of 2-fold change and *P*-value < 0.05.

Table 5.2 The top 10 up-regulated and down-regulated genes in granulosa cells from preovulatory follicle (24 h post-LH) of aged cows (compared to their younger daughters; ≥ 2-fold change in expression; $P \leq 0.05$), as assessed by microarrays.

Expression	Gene Symbol	Protein encoded	Fold Change
Up-regulated	TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	4.8
	GEM	GTP binding protein overexpressed in skeletal muscles	4.5
	CRISPLD2	Cysteine-rich secretory protein LCCL domain containing	4.3
	GFRA1	GDNF family receptor alpha 1	3.5
	NUDT11	Nudix (nucleoside diphosphate linked moiety X)-type motif 11	3.5
	MRO	Maestro	3.4
	ASB9	Ankyrin repeat and SOCS box containing 9	3.4
	CLDN11	claudin 11	3.2
	SLC39A8	Solute carrier family 39 (zinc transporter), member 8	3.0
	GFPT2	Glutamine-fructose-6-phosphate transaminase 2	3.0
Down-regulated	VNN1	Vanin 1 or Vascular non-inflammatory molecule 1	-4.4
	PERP	TP53 apoptosis effector	-4.4
	PPL	Periplakin	-4.4
	EPCAM	epithelial cell adhesion molecule	-4.2
	KRT19	keratin 19	-4.0
	FXYD3	FXYD domain containing ion transport regulator 3	-4.0
	TMEM79	transmembrane protein 79	-3.9
	WWC1	WW and C2 domain containing 1	-3.8
	GIPC2	GIPC PDZ domain containing family, member 2	-3.8
	DSG2	desmoglein 2	-3.6

5.3.3 Functional classification of transcripts

Analysis of molecular and cellular functions revealed that cell death, cellular development, tissue development, cell morphology, cellular assembly and organization, cellular movement, inflammatory response, immune cell trafficking were decreased ($P < 0.05$) while nucleic acid metabolism increased in granulosa cells of aged vs. young cows (Fig. 5.4A). Similarly, analysis of canonical pathways revealed that glutathione metabolism, mitochondrial dysfunction, oxidative phosphorylation, NRF-2 mediate oxidative stress response, protein kinase A signaling and HIF1α signaling were the most significant ($P < 0.05$) pathways that were affected in aged cows vs. young cows (Fig. 5.4B).

5.3.4 Upstream regulators

Based on expression of genes in the dataset, pathway analysis generated a list of potentially "inhibited" and "activated" upstream regulators (Table 5.3) that can explain the observed gene expression changes in the granulosa cells. About 80% of the upstream molecules were proposed to be "activated" in aged cows vs. young cows and included follicle stimulating hormone (FSH), FSH receptors (FSHR), Luteinizing hormone (LH), human chorionic gonadotropin hormone (hCG), Myelocytomatosis Viral Oncogene Homolog C (MyC), Myelocytomatosis Viral Oncogene Homolog B (MyB), Prostaglandin E2, Insulin-like growth factor 1 receptor (IGFIR), cyclic AMP. Suggested "inhibited" upstream regulators included estrogen receptor and inhibin alpha (INHA). Potential upstream regulators were included in the network as core molecules to explain downstream changes in gene expression (Fig. 5.5).

5.3.5 Networks analyses

Network analysis of the genes relevant to reproductive physiology and response to luteinizing hormone revealed that the several genes in granulosa cells of aged cows did not follow the known spatiotemporal pattern and were still up-regulated 24 h after LH surge (Fig. 5.5). Up- and down-regulated genes in the network were related to 1) response to gonadotropin (RGS2, SERPINE2, PTGS2), 2) granulosa cell differentiation and luteinization (CTGF, TNFAIP6, GADD45B, VNN1) and 3) steroidogenesis (STAR, HSD3B2, FDX3, NR5A2, NR4A1).

5.3.6 Validation of differentially expressed transcripts via RT-qPCR

RT-qPCR data of six transcripts (VNN1, GADD45B, NR5A2, TNFAIP6, RGS2, and SERPINE2) was analyzed (n=4 aged cows, 5 young cows) to validate microarray analysis. Fold differences demonstrated that five transcripts (VNN1; $P \leq 0.05$), (GADD45B; $P \leq 0.01$), (TNFAIP6; $P \leq 0.01$), (NR5A2; $P \leq 0.01$) and (RGS2; $P \leq 0.01$) showed differences similar to expression pattern as microarrays when compared between aged vs. young cows (Fig.5.6). Fold difference for SERPINE2 did not reach the set statistical significance level (0.05) but showed a tendency for a difference ($P < 0.1$) with an expression pattern similar to microarray data.

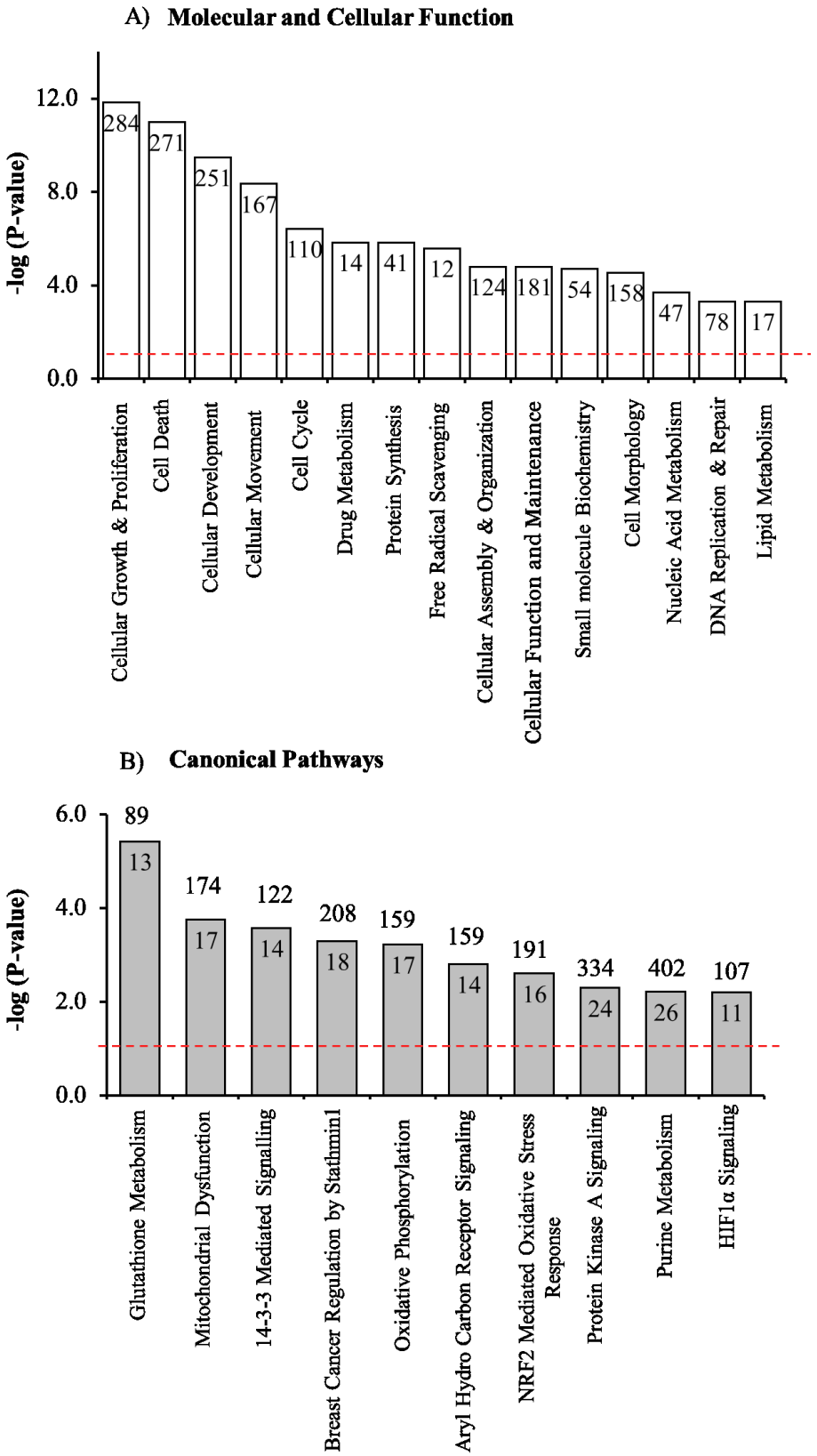
A) Molecular and Cellular Function
-log (P-value)
12.0
8.0
4.0
0.0
284
271
251
167
110
14
41
12
124
181
54
158
47
78
17
Cellular Growth & Proliferation
Cell Death
Cellular Development
Cellular Movement
Cell Cycle
Drug Metabolism
Protein Synthesis
Free Radical Scavenging
Cellular Assembly & Organization
Cellular Function and Maintenance
Small molecule Biochemistry
Cell Morphology
Nucleic Acid Metabolism
DNA Replication & Repair
Lipid Metabolism
B) Canonical Pathways
-log (P-value)
6.0
4.0
2.0
0.0
89
13
174
17
122
14
208
18
159
17
159
14
191
16
334
24
402
26
107
11
Glutathione Metabolism
Mitochondrial Dysfunction
14-3-3 Mediated Signalling
Breast Cancer Regulation by Stathmin1
Oxidative Phosphorylation
Aryl Hydro Carbon Receptor Signaling
NRF2 Mediated Oxidative Stress Response
Protein Kinase A Signaling
Purine Metabolism
HIF1α Signaling

Figure 5.4 Molecular and cellular function analysis (A) and canonical pathway analysis (B) of transcripts differentially expressed in granulosa cells of preovulatory follicles of aged vs. young cows. The number inside each bar represents the number of transcripts associated with the given function or pathway. The number at the outside of each bar in the canonical pathway (B) represents the total number of transcripts known to be associated with that specific pathway. The horizontal axis represents various molecular functions (A) and pathways (B). The vertical axis represents the level of significance in the degree of each function or pathwsy in aged vs. young cows at or above the threshold cut-off of $P < 0.05$ (equal to –log 1.30, red dotted line). A taller bar represents a greater level of statistical significance. Graphs generated by Ingenuity Pathway Analysis software.

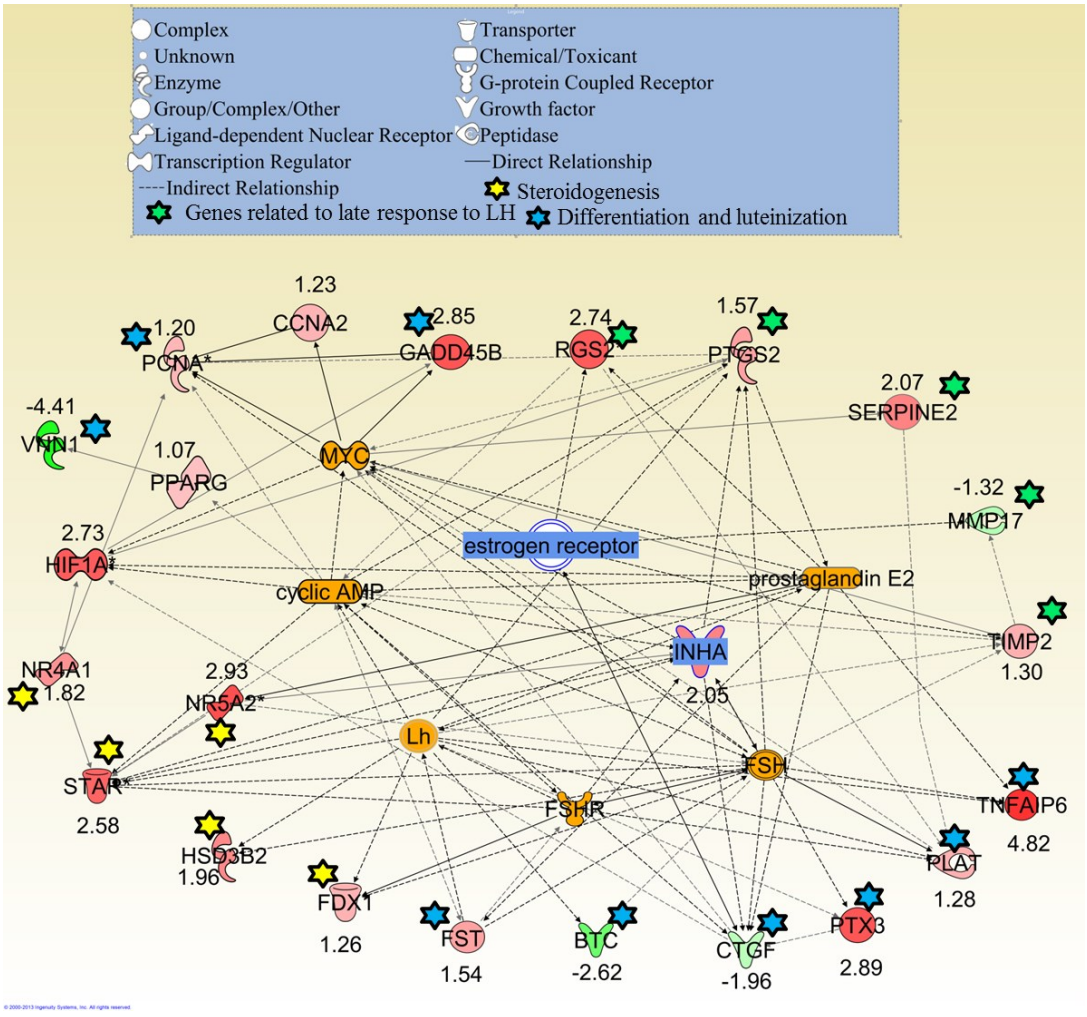

Figure 5.5 Network of differentially expressed genes (represented as nodes; red and green) and biological relationship between genes (lines) in preovulatory follicle of aged cows (Reference group: young cows; daughters of aged cows). Relationship-lines are supported by al least one reference derived from Ingenuity Knowledge Base. Upstream regulators ("Activated"; organe colored): cyclic AMP, FSH, FSHR, LH, MYC, prostaglandin E2 and "Inhibited" (blue-colored): estrogen receptor, INHA) were identified by the Ingenuity Pathway Analysis software (Table 5.3) and were placed in the centre of the network. The color-intensity of each node indicates the degree of up- (red) or down- (green) regulation of a gene expression at the $\log_2$ of fold-change (shown next to each gene) in aged cows relative to young cows. Gene symbols are extracted from Entrez Gene or UniGene (www.ncbi.nlm.nih.gov/unigene). Illustration generated by Ingenuity Pathway Analysis software.

Table 5.3 Upstream regulators predicted by Ingenuity pathway analysis (IPA) software to be activated (increased influence on downstream targets) or inhibited (decreased influence) based on expression of target molecules identified in granulosa cell of the preovulatory follicle (24 h post-LH) from aged (Reference group: young cows). Bold face symbols are known for their role in ovarian functions. Upward and downward arrows indicate up-regulation and down-regulation of the transcripts.

Upstream Regulator	Molecule Type	Predicted Activation State	Activation Score*	P-value	Target molecules in the dataset (Gene symbols)
Estrogen Receptor	Group	Inhibited	-3.34	1.86×10^{-8}	↓***BMP7***, ↑*CDH12*, ↓*CLDN4*, ↓*CLN8*, ↑*COL4A1*, ↑*COL4A2*, ↓***CTGF***, ↑*DCHS1*, ↓*DSP*, ↓*ERBB3*, ↓*F11R*, ↓***FGFR2***, ↑*GSTA5*, ↑*HSP90AA1*, ↓*JAG1*, ↓*KRT18*, ↓*KRT19* , ↓*KRT7*, ↓*KRT8*, ↓*LAMC2*, ↓***LTF***, ↓***MMP17***, ↓*MUC1*, ↑*PCDH18*, ↑***RGS2***, ↓*TFF3*, ↓***TGFB3***, ↑***TGFBR3***, ↑***TIMP2***, ↑***VEGFC***, ↑***VIM***
INHA	Growth factor	Inhibited	-2.72	5.45×10^{-5}	↓***CTGF***, ↓*CYR61*, ↓*F11R*, ***FST***, ***INHA***, ↑*JAM2*, ↓*KRT18*, ↓*KRT8*, ↑*MATN2*, ↓***PIK3IP1***, ↑***PTGS2***, ↑***PTX3***, ↑*SMARCA1*, ↑***STAR***, ↑***TPM2***
LH	Complex	Activated	2.07	1.27×10^{-8}	↓*ARHGEF5*, ↓*ARL4C*, ↑*BNIP3L*, ↓*BTC*, ↑*CAPZA1*, ↓*CARD10*, ↓*CASP4*, ↑***CHUK***, ↓***CREBL2***, ↓*EZR*, ↑***FDX1***, ↑*FKBP5*, ↑***FST***, ↑*GEM*, ↑***HSD3B1***, ↑***INHA***, ↑*ITPR1*, ↓*KRT18*, ↑***MSMO1***, ↓***PAWR***, ↑*PGK1*, ↑***PLAT***, ↑*PRKD1*, ↑***PTGS2***, ↓*PTPRF*, ↓*SH3BP4*, ↑***STAR***, ↓***TFPI2***, ↑***TNFAIP6***, ↑***TPM2***, ↑***TUBA1A***, ↑***VCL***, ↑***VEGFC***,
Arachadonic acid	Chemical-endogenous	Activated	2.12	2.05×10^{-4}	↓*AQP3*, ↓*CLU*, ↓*FADS1*, ↑***PLA2G4A***, ↑*PRKD1*, ↑***PTGS2***, ↑***RGS2***, ↑***SCD***, ↑*SOAT1*, ↑***STAR***, ↑***VIM***
FSHR	G-protein coupled receptors	Activated	2.21	5.55×10^{-3}	↑***CLDN11***, ↑***FDX1***, ↑***GATA4***, ↑***HSD3B1***, ↑***INHA***, ↑***PLAT***, ↑***STAR***
MYB	Transcription Regulator	Activated	2.40	5.84×10^{-3}	↓*ANPEP*, ↓***CDKN2B***, ↓*GSTM1*, ↑*HSPA8*, ↑*ITPR1*, ↑*MAT2A*, ↑***PCNA***, ↑***PTGS2***, ↑***VIM***
MYC	Transcription regulator	Activated	2.42	1.01×10^{-8}	↑***ANXA5***, ↓***APP***, ↑*ARL6IP1*, ↑*BUB1*, ↑*CAPN2*, ↑***CCNA2***, ↑*CD48*, ↓*CD9*, ↓***CDKN2B***,***CDKN2D***, ↓*CLU*, ↑*CNP*, ↑*COL4A1*, ↑*COL4A2*, ↑*COX5B*, ↑*COX6A1*, ↓*CXCL10*, ↑*CYCS*, ↑*EIF2S1*, ↑*EIF2S2*, ↑*ENO1*, ↓*EPCAM*, ↑*FAM129A*, ↓*FBN1*, ↓***FOXA2***, ↑***FSTL1***, ↑***GADD45B***, ↑***GATA4***, ↑*GCLM*, ↑*GCSH*, ↓*GPC1*, ↑***HIF1A***, ↑*HNRNPH1*, ↑*HSP90AA1*, ↑*HSPD1*, ↑ ***ID2***, ↓*IRF7*, ↑***LDHA***, ↑*LGALS1*, ↑*LXN*, ↑*MAN2A1*,

					↑*MAT2A*, ↑*MYO1B*, ↓*NDRG1*, ↑*NDUFS4*, ↑*NGFRAP1*, ↑***PCNA***, ↓*PDK1*, ↓*PERP*, ↑*PGK1*, ↑*PMP2*, ↑*POLR1B*, ↑*PPIA*, ↑*PPP1CC*, ↑***PRDX3***, ↑*PRDX4*, ↑*PTN*, ↑*RPL35*, ↑*SHMT1*, ↑*STMN1*, ↑*SUCLA2*, ↑*TIMM23*, ↑***TIMP2***, ↑*TMSB10*, ↑*Tpi1*
Cyclic AMP	Chemical-endogenous	Activated	2.49	$1.77x10^{-2}$	↑*ANTXR2*, ↓***APP***, ↓***EPCAM*,** ↑***GATA4***, ↑*GCH1*, ↑***HIF1A***, ↓*IL2RA*, ↑***INHA*,** ↓***PDE4B***, ↑*PLA2G4A*, ↑*PTCH1*, ↑***PTGS2*,** ↑***STAR***, ↑*YWHAG*
hCG	Complex	Activated	2.58	$3.27x10^{-7}$	*ABCB1*, ↓*APCDD1*, ↓***BTC***, ↓*CLU*, ↓*CXCL10*, ↑***FST***, ↑***GATA4***, ↓*GJB2*, ↑***HIF1A*,** ↑***HSD3B1*,** ↑***INHA***, ↑*MRPS6*, ↑***NR4A1*,** ↑***NR5A2***, ↑***NRP1*,** ↑***PCNA***, ↑*PKIA*, ↑***PLAT***, ↑*PPARG*, ↓*PRSS2*, ↑***PTGS2*,** ↑***PTX3*,** ↑***RGS2*,** ↑***SMARCA1***, ↑***SMARCA5***, ↑***SPRY2*,** ↑***STAR***, ↑*STEAP1*, ↓*TFPI2*, ↑***TNFAIP6*,** ↑***VEGFC*,** ↑*YWHAG*
PGE2	Chemical-endogenous	Activated	2.79	$1.35x10^{-4}$	↓***APP***, ↓*ATF3*, ↓***CTGF***, ↓*CXCL10*, ↓*ENPP3*, ↓*FOSB*, ↑***FST*,** ↑***FSTL3***, ↓*GZMB*, ↑***HIF1A***, ↑***IGFBP7***, ↓*IL2RA*, ↑***NR4A1*,** ↑***NR5A2*,** ↑***NRP1*,** ↓***PDE4B***, ↑***PLA2G4A***, ↑***PPARG*,** ↑***PTGS2*,** ↑***RGS2***, ↓*S100A8*, ↑***STAR*,** ↑***TGFBR3*,** ↑***TNFAIP6*,** ↑***VEGFC***
IGFlR	Trans-membrane receptor	Activated	2.91	$3.51\ x10^{-4}$	↑*ACADM*, ↓*ANXA3*, ↑*ATP5A1*, ↑*ATP5B*, ↑*ATP5J*, ↑*CD48*, ↑*CD5L*, ↓*CLU*, ↑*COL4A1*, ↑*COL4A2*, ↑***HIF1A*,** ↑*ID2*, ↑*IGF2*, ↑*KIF20A*, ↑*NDUFV2*, ↑***PLAT*,** ↑***PTGS2***, ↑*SCG2*, ↑***TPM2***, ↑***VEGFC***
FSH	Complex	Activated	3.02	$8.85x10^{-9}$	↓*AQP3*, ↓***ARHGEF5***, ↓*ARL4C*, ↑*BNIP3L*, ↑*CAPZA1*, ↓*CARD10*, ↓*CASP4*, ↑***CHUK***, ↑*COPS5*, ↓***CREBL2*,** ↓***CTGF***, ↑*DHX15*, ↓***EFNA1*,** ↓*EZR*, ↑***FDX1***, ↑*FKBP5*, ↑***FST*,** ↑***GATA4***, ↑*GEM*, ↑***HSD3B1***, ↑***INHA***, ↑*ITPR1*, ↓*KRT18*, ↓*LYPD3*, ↑***MSMO1***, ↑***NR4A1***, ↓***PAWR***, ↑***PCNA*,** ↑*PGK1*, ↑***PLAT***, ↑*PRKD1*, ↑***PTGS2***, ↓*PTPRF*, ↓*SH3BP4*, ↑***STAR***, ↓***TFPI2*,** ↑***TGFBR3*,** ↑***TIMP2***, ↑***TNFAIP6*,** ↑***TPM2*,** ↑***TUBA1A*,** ↑***VCL***, ↑***VEGFC***

Threshold score (±2.00) used to identify activated and inhibited upstream regulators.

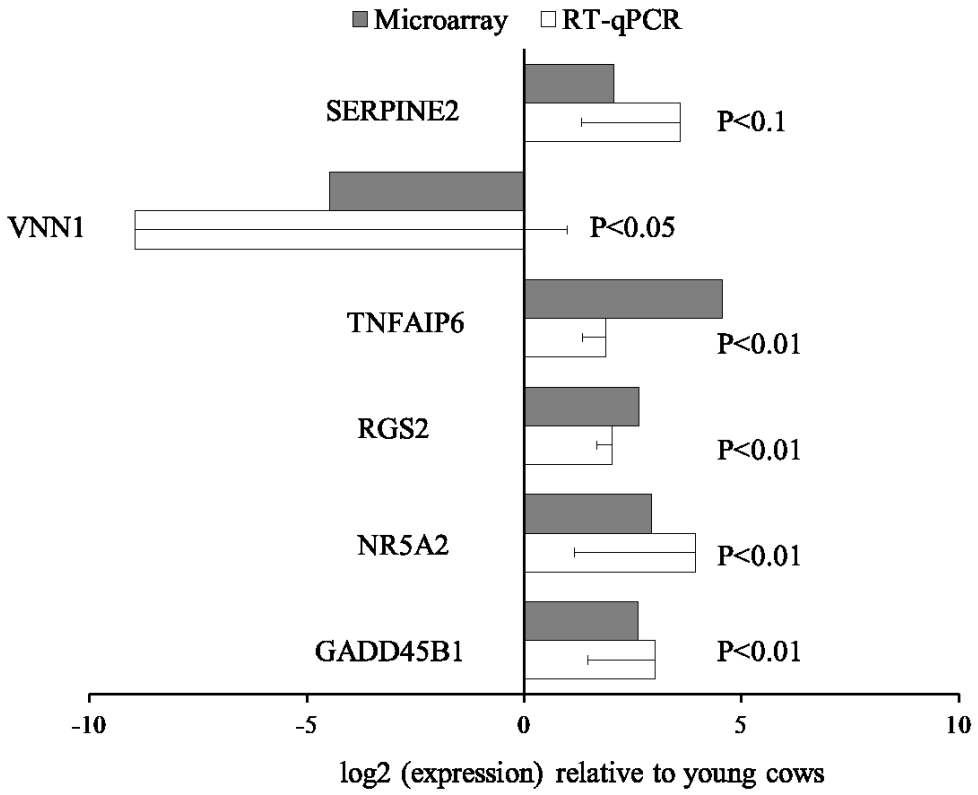

Figure 5.6 Expression of granulosa cell transcripts (log_2 of fold change) in aged cows relative to young cows. Open bars with standard error (capped line) represent the expression of the transcripts obtained by RT-qPCR analysis (granulosa cell samples from n=4 aged, n=5 young cows). Solid bars represent expression of same transcripts obtained by microarray analysis (granulosa cell samples from n=3 aged and n=3 young cows). Differences in the expression of transcripts between aged and young cows in RT-qPCR analyses are denoted by *P*-values.

5.4 Discussion

This study highlights how age-associated changes in granulosa cell transcripts can influence the gonadotropin-mediated ovulatory process in aged cows. Results suggest that the genes in granulosa cell from aged cows did not follow the expected temporal pattern of gene expression as compared to their daughters 24 h after LH treatment (Fig. 5.7). As a result, preovulatory follicle from aged cows showed decreased intrafollicular concentrations of progesterone, slow differentiation and luteinization, and delayed ovulation. Considering the mRNA expression of genes in granulosa cells, potential intracellular mechanisms of delayed ovulation in aged cows may involve 1) post-receptor desensitization or signal attenuation of G-coupled protein receptors (GPCR) due to late induction of RGS2, 2) inactivation of tissue plasminogen activator by serine proteinase inhibitors (SERPINE2) and the tissue metalloproteinase (MMP), and 3) decreased production of intrafollicular progesterone that leads to delayed production of prostaglandin E2 via PTGS2 and matrix destabilization via TNFAIP6 (Fig. 5.8).

In the current study, interval to ovulation after PGF_2 alpha administration was prolonged by 25 h in aged (115 h) vs. young (90 h) cows. This prolongation is more likely to be due to increase in the interval between LH surge to ovulation as concluded by an earlier report (Malhi *et al.*, 2008) in which higher proportions of aged (13%) than young (4%) cows ovulated late, during 48-72 h, after LH administration. Another previous report indicated that peak ovulatory plasma LH concentrations did not differ ($P = 0.4$) between aged cows and their daughters (Malhi *et al.*, 2005). The findings from this study and previous reports indicate that the ovulatory process is impaired or delayed in aged cows. Therefore, we challenged the granulosa cells of preovulatory follicles from aged and young cows with the single dose of LH to determine

their preparedness for ovulation. Intrafollicular concentrations of progesterone, 24 h after LH treatment, were lower ($P < 0.05$) while estradiol concentrations were higher ($P < 0.05$) in aged cows compared to the young cows (Fig. 5.2). These findings should be interpreted after taking into account the previously reported biphasic increase of intrafollular progesterone in cattle after 1.5 h and 23 h of LH surge (Fortune *et al.*, 2009). Low plasma concentration of progesterone in during preovulatory follicle development leads to persistent follicles, premature initiation of meiosis in the oocyte and early embryonic mortality in cattle (Inskeep, 2004). Also, oocyte competence and (Aardema *et al.*, 2013) subsequent luteal function (McNatty, 1979) are related to progesterone concentration of preovulatory follicle. In this context, decreased level of intrafollicular progesterone 24 h after LH treatment support the hypothesis of an inefficient synthesis of progesterone in aged cows and may be a reason for subsequent lower luteal phase progesterone concentrations and fertility in aged cows (Malhi *et al.*, 2005; Malhi *et al.*, 2007).

Analysis of several gene transcripts (Fig. 5.7) reveals the changes in granulosa cells of aged vs. young cows were proceeding late in response to exogenous LH treatment after 24 h. It is interesting to note that granulosa cell response was delayed in aged cows despite no difference in the microarray expression of LH receptors were recored between the two age groups. Ovulatory process depends upon proteolytic activity induced by the serine proteases (urokinase; uPA or tissue plasminogen activators; tPA), which are regulated by SERPINE2 (Bedard *et al.*, 2003). Previously, it has been documented that mRNA of SERPINE2 decreased rapidly in bovine granulosa cells post-LH or hCG treatment (Dow *et al.*, 2002; Bedard *et al.*, 2003; Ndiaye *et al.*, 2005). However, in the current study, higher mRNA of SERPINE2 in granulosa cells of aged vs. young cows differ from findings of the previous studies (Dow *et al.*, 2002; Bedard *et al.*, 2003; Ndiaye *et al.*, 2005) and, therefore, suggest that granulosa layer contributions to the uPA or tPA

activity in thecal connective tissue may be markedly decreased in aged cows and may cause a delay in the ovulatory process (Fig.5 8).

In granulosa cells of the ovulatory follicle, regulator of G-protein signaling-2 (RGS2) is transcribed in response to ovulatory dose of LH to regulate membrane signaling (Ujioka *et al.*, 2000). RGS2 accelerates the rate of GTPase activity to hydrolyze GTP to GDP on the alpha subunit of an already activated G-protein (Kehrl & Sinnarajah, 2002) and in turn deactivates the G-alpha subunit and signal transduction (Berman & Gilman, 1998; Wu *et al.*, 2008). Consequently, duration and intensity of gonadotropin signals through G-protein coupled receptors is impeded along with the decrease in adenylyl cyclase activity in gonadotropin responsive cells (Berman & Gilman, 1998; Kehrl & Sinnarajah, 2002). Previously, in rat and mouse, it has been demonstrated that, in response to hCG, expression of RGS2 protein increased in mural granulosa cells by 2 h, moved towards cumulus cells by 12 h after hCG treatment and finally faded away by 24 h (Ujioka *et al.*, 2000; Espey & Richards, 2002). Similarly, another study demonstrated that mRNA levels of RGS2 in granulosa cell lines of human (KGN) and mouse (NT-1) increased after 2 h and 6 h of hCG treatment, respectively, before returning to basal levels by 24 h (Wu *et al.*, 2008). In this context, mRNA levels of RGS2 in granulosa cells of young cows suggest a timely down-regulation of RGS2 mRNA levels at 24 h, allowing LH to initiate necessary ovulatory changes in the cells. On the other hand, although the granulosa cells of aged cows were exposed to exogenous LH for 24 h, but the cells represent an early stage of increase in mRNA levels of RGS2 expression in response to LH treatment (2.7 and 2.0-fold higher expression than young cows in microarray and RT-qPCR data). In support, adenylyl cyclase expression (ADCY5/6) also decreased in granulosa cells of aged cows. Hence, we infer that post-receptor desensitization or signal attenuation of GPCR in granulosa cells of aged cows

due to late induction of RGS2 may be associated with delayed ovulation (Fig. 5.8) (Sasson *et al.*, 2004).

Late changes in transcripts of granulosa cells after LH surge were also evident from higher mRNA levels of PTGS2 *(*Cyclooxygenase 2*)* in aged cows than young cows. In cultured bovine granulosa cells, mRNA levels of *PTGS2* increased from 2 to 6 h after LH treatment, and were maintained until 18 h but decreased by 24 h after LH (Monga *et al.*, 2011). Another report showed an increase mRNA expression of PTGS2 in bovine granulosa cells only after18 h of hCG treatment (Sirois *et al.*, 2004). In this context, mRNA levels of PTGS2 in granulosa cells of aged cows are analogous to 6 or 18 h post-LH (1.6 fold higher expression than young cows in microarray). In contrast, decreased mRNA expression of PTGS2 in granulosa cells of young cows (24 h after LH treatment) indicates well-timed granulosa cell transition from preovulatory to luteinized phenotype (Monga *et al.*, 2011). PTGS2 has also been proposed as a biomarker of impending ovulation because of the consistent interval between its induction to ovulation (about 10 h) in horse, rat and cattle (Richards, 1997; Liu & Sirois, 1998). This suggests that late induction of PTGS2 (higher mRNA at 24 h post-LH) in granulosa cells of aged cows may delay the ovulation (Fig. 5.8).

The late induction of PTGS2 in granulosa cell of aged cows also suggests an on going process of prostaglandins production which is substantiated by the active status of endogenously produced upstream molecule prostaglandin E2 (PGE2) and arachidonic acid in granulosa cells (LeMaire *et al.*, 1973)(Table 3). The late induction of PTGS2 in granulosa cells 24 h after LH treatment may be associated with the decreased progesterone concentrations (Bridges *et al.*, 2006) in aged cows (Fig. 5.2). In addition, both age related higher plasma basal FSH concentrations (Loh *et al.*, 2002) and predicted active status of endogenous FSH receptors

(FSHR) in granulosa cells of aged cows (Table 5.4) indicate a protracted LH-mediated transition of granulosa cells from preovulatory (↑FSHR, ↑PTGS2) to luteinized phenotype (↓FSHR and ↓PTGS2) (Monga *et al.*, 2011). The acquisition of LH receptors (LHR) by the follicular cells of the dominant follicle allows it to survive despite decline in plasma FSH level at the time of selection. Despite significantly higher basal FSH concentrations throughout the cycle due to lack of negative feedback mechanism from granulosa cells (Khan *et al.*, 2012), aged cows maintain temporal association of FSH with the follicle selection (Malhi *et al.*, 2005). In preovulatory follicles from aged cows, endogenously active FSHR indicate that the response of granulosa cells to FSH may not be truncated even 24 h after LH treatment.

After the ovulatory surge of LH, luteinization of granulosa cells begins and is marked with increased production of progesterone than estradiol (Fortune & Quirk, 1988). In current study, several genes encoding enzymes and transcription factors related to steroidogenesis (STAR, NR4A1, HSD3B1, and NR5A2*)* were up-regualted in granulosa cells of aged than young cows. Expression of these genes corresponds to the early response of granulosa cells to LH. For example, NR4A1 has been shown to induce rapidly after 15 min to 2 h after LH/hCG in granulosa cells of mouse and buffalo (Carletti & Christenson, 2009; Monga *et al.*, 2011). Higher expression of NR4A1 has been suggested in steroidogenic shift from estrogen to progesterone synthesis by repressing aromatase *(*CYP19A1*)* (Wu *et al.*, 2005) and upregulating HSD3B1 expression in granulosa cells (Havelock *et al.*, 2005). Similarly, NAR5A2 has been documented for cellular growth, steroidogenesis, cholesterol metabolism and ovulation (Parker & Schimmer, 1997; Liu *et al.*, 2003; Fayad *et al.*, 2004; Duggavathi *et al.*, 2008). Transcripts of NR5A2 decreased gradually by 24 h of LH surge and between 30-39 h after hCG treatment of granulosa cells of pigs and horses, respectively (Boerboom *et al.*, 2000; Agca *et al.*, 2006). Considering the

gonadotropin induced transient mRNA expression of STAR in rat (Espey & Richards, 2002) and cattle (Gilbert *et al.*, 2011), increased mRNA expression of *STAR* in aged cows indicates a late peak/level by 24 h after LH and is contrary to the decreased mRNA expression of STAR in young cows who may have already begun luteinization process by 24 h after LH. In this context, granulosa cells 24 h after LH treatment in aged cows show a late change from estradiol to progesterone synthesis as compared to young cows (Fig. 5.8).

In agreement, intrafolliclicular progesterone concentrations were lower ($P < 0.05$) whereas estrogen concentrations were higher in aged cows than middle-aged and young cows (Fig. 5.2). Similar findings have been reported in aged women where progesterone production in granulosa cells decreased and estradiol concentration increased with advanced age (Pacella *et al.*). This delay in steroidogenic shift from estrogen to progesterone synthesis prominently suggests decreased potential of progesterone synthesis from subsequent developing corpus luteum. In support, bovine model of reproductive aging has been characterized for lower luteal phase plasma progesterone concentrations in aged cows than their daughters (Malhi *et al.*, 2005). Consistent with the higher intrafollicular concentrations of estradiol in this study, higher plasma concentrations of estrogen has been documented during the preovulatory period in aged cows vs. their daughter (Malhi *et al.*, 2005). Also, in the current study, high intrafollicle concentrations of estrogen may be the cause of increase in NRP1 transcripts, which has otherwise been shown to down-regulate in post-LH bovine granulosa cells (Shimizu *et al.*, 2006). These findings suggest a compromised steroidogenic process in granulosa cells of aged cows 24 h after LH.

GADD45B is involved in multiple functions such as differentiation (Zhang *et al.*, 1999; Mihm *et al.*, 2008), luteinization, ovulation (Sriraman *et al.*, 2008), and cell cycle inhibition (Abdollahi *et al.*, 1991; Vairapandi *et al.*, 2002). High mRNA expression of GADD45B in

granulosa cells of aged cows 24 h after LH treatment could be due to the activation of upstream molecule MYC (as recommended by IPA), which is known for controlling transcription of growth related genes (Dang, 1999). In mouse, the expression of GADD45B gene up-regulated within 1 h after hCG but declined to basal level by 4 h after hCG (Carletti & Christenson, 2009). High mRNA expression of GADD45B in bovine dominant follicles has been suggested supporting antiapoptotic activity (Mihm *et al.*, 2008). It looks as if the higher GADD45B mRNA level in granulosa cells of aged cows is related to the decreasing frequency of cell death/apoptosis as recommended by the functional analysis (Fig. 5.4B), and indicated by decreased expression of proapoptotic gene PAWAR as well (Fig. 5.5). The proliferative nature of granulosa cells is substantiated by the increased mRNA levels of PCNA, E2F5 transcription factor, and CCNA2 (Fig. 5.5) as well as from functional analysis of the transcripts in aged cows (Fig. 5.4). Taken together, these results suggest slow differentiation of granulosa cell of aged cows after LH surge.

In the current study, increased mRNA of TNFAIP6 and decreased mRNA of VNN1 24 h after LH treatment in aged cows also suggest late differentiation and luteinization of granulosa cells. Spatio-temporal expression of TNFAIP6 mRNA in bovine granulosa cells showed a rapid increase in its expression from 2 to 6 h post-LH/hCG (Sayasith *et al.*, 2008) followed by decrease in expression at 22 h post-LH (Gilbert *et al.*, 2011). Likewise, mRNA levels of VNN1 increased in mouse granulosa cells during early stages of luteinization after hCG treatment (McRae *et al.*, 2005) TNFAIP6 via cAMP/PKA pathway is critical for the stabilization of extracellular matrix (Jessen & Odum, 2003) and regulates migration of inflammatory cells before ovulation (Sayasith *et al.*, 2007). VNN1 has been shown to be involved in corpus luteum development and cell migration into developing tissue (McRae *et al.*, 2005). Likely, reasons for

higher expression of TNFAIP6 may be the late activation of PGE2 and cAMP molecules (Sayasith *et al.*, 2007) as suggested by IPA (Table 5.3; Fig. 5.5). Consequently, decreased mRNA levels of VNN1 indicate decreased inflammatory changes (Berruyer *et al.*, 2004) in aged cows and will possibly cause a delay in follicle maturation and ovulation. In this context, it is reasonable to assume that granulosa cells of aged cows are differentiating and luteinizing late 24 h after LH treatment.

In conclusion, the study of the gene transcripts from granulosa cells of preovulatory follicles at 24 h after LH between aged and young cows reveals changes in transcripts related to 1) late LH response (↑RGS2, ↑SERPINE2, ↑PTGS2), 2) delayed differentiation and luteinization of granulosa cells (↑TNFAIP6, ↑GADD45B, ↓VNN1), sub optimal progesterone synthesis (↑STAR, ↑HSD3B2, ↑NR5A2, ↑NR4A1). Detected changes in cellular pathways were supportive of detected higher intrafollicular estradiol and lower progesterone concentrations. Our hypotheses that 1) the ovulatory dysfunction in aged cows is associated with modified expression of transcripts in granulosa cell of preovulatory follicles 24 h after LH treatment and 2) the ability of the granulosa cells to synthesize progesterone, in response to LH, decreases with age, were supported. Delay in ovulation may likely be associated with 1) post-receptor desensitization or signal attenuation of GPCR 2) inactivation of tissue plasminogen activator and, 3) decreased production of intrafollicular progesterone leading to delayed production of prostaglandin E2 by granulosa cells of aged cows. Alterations in these molecular mechansisms may result in compromised oocyte competence, a delayed or decreased ovulatory response in aged cows, sub-optimal corpus luteum development and subsequently early embryonic losses in aged cows. Results from this study provide the basis to optimize clinical treatments in aged humans and animals to improve the outcome of assisted reproductive techniques.

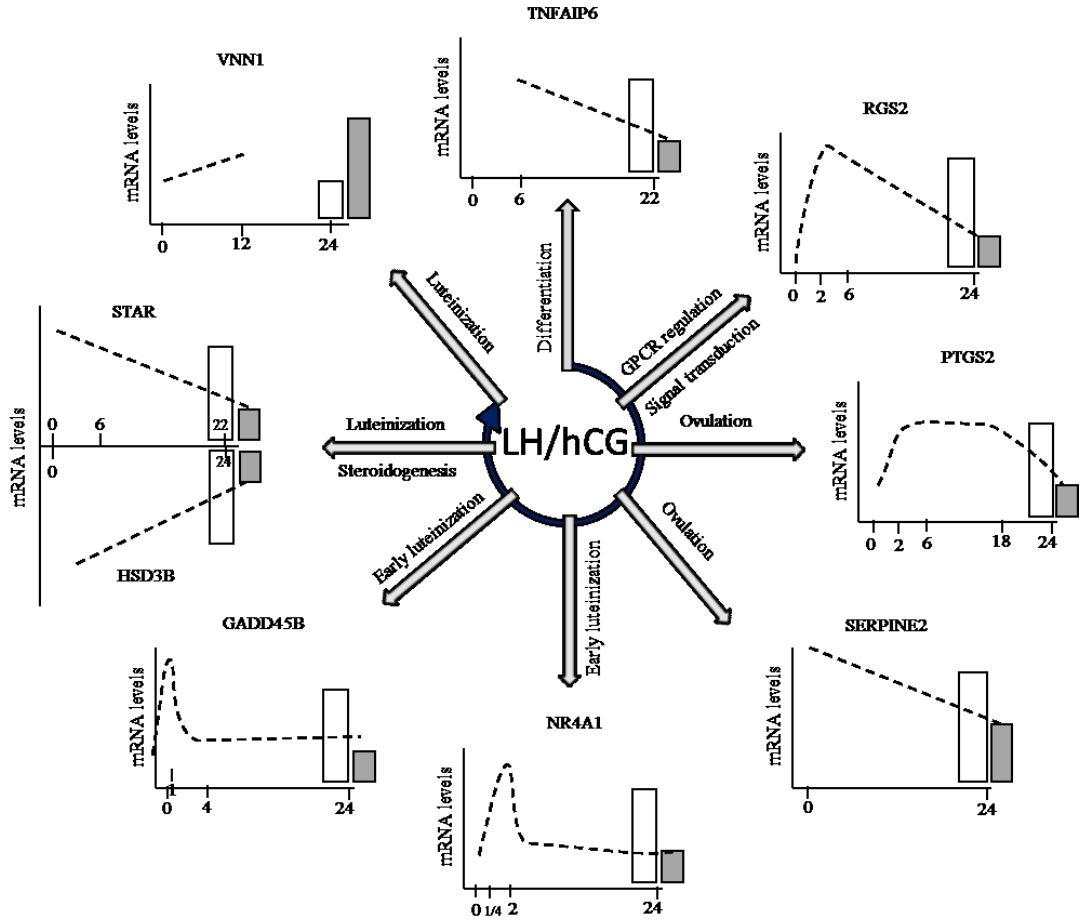

Figure 5.7 Preovulatory response in gene expression of granulosa cells after administration of LH or hCG. Temporal changes (dotted lines) are extrapolated from reports of other granulosa cell studies in cattle, human, mouse, and rat (see Discussion). Differences between aged and young cows at 24 h (open and solid bars, respectively) are microarrays data from the present study. The horzontal axis represents time (h) after LH or hCG administration. The vertical axis represents arbitrary units of mRNA levels in the granulosa cells.

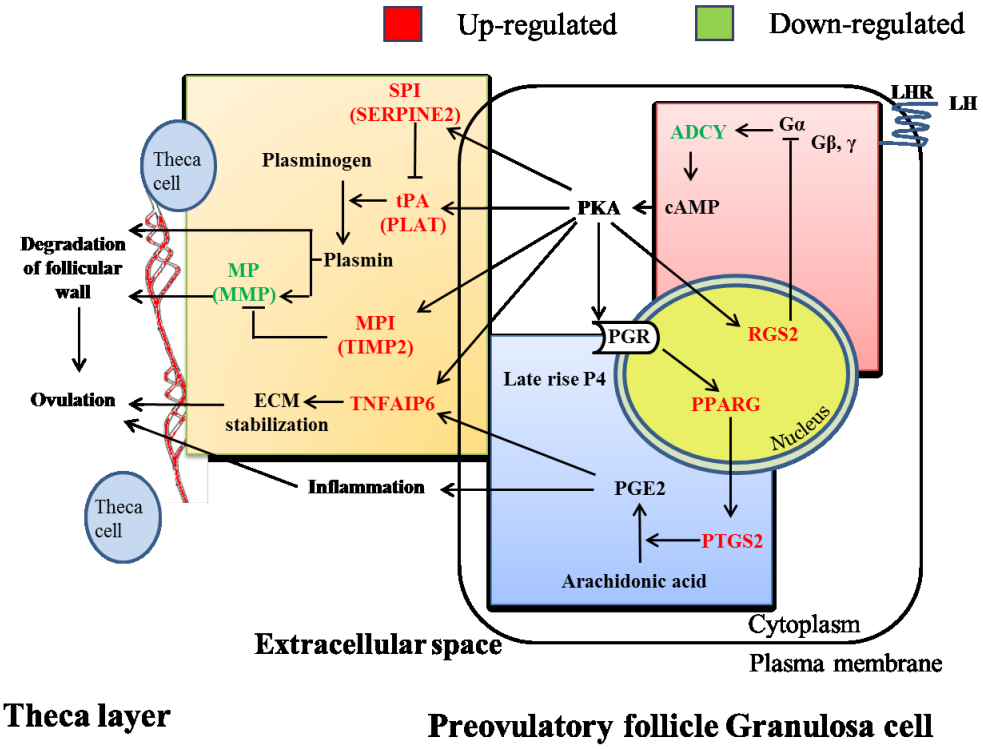

Figure 5.8 Schematic diagram of a granulosa cell of a preovulatory follicle 24 h after LH depicting the potential mechanism of delayed ovulation in aged cows. 1) Post-receptor desensitization of G-alpha subunit by regulator of G-protein signaling 2 (RGS2). 2) Blocking of tissue plasminogen activator; (PLAT) by serine proteinases inhibitors (SERPINE2) and tissue metalloproteinase (TIMP2). 3) Late rise in progesterone leading to delayed production of prostaglandin E2 (PGE2) via prostaglandin synthase 2 (PTGS2), and matrix destabilization via tumor necrosis factor alpha induced protein 6 (TNFAIP6). The red color indicates up-regulated transcripts and green indicates down-regulated transcripts in aged cows relative to the young cows. Arrows point in the direction of the reaction while capped lines indicate a blocking action. Abbreviations: Leutinizing hormone receptor (LHR), Adenylyl cyclase (ADCY), cyclic Adenosine Mono Phosphate (cAMP), Protien kinase A (PKA), Peroxisome proliferator-activated receptor gamma (PPRG); extracellular matrix (ECM), Martix metalloproteinases (MMPs).

6 CHAPTER 6: CHANGES IN TRANSCRIPTOME OF GRANULOSA CELLS BETWEEN THE TIME OF DOMINANT FOLLICLE SELECTION AND 24 H POST LH IN YOUNG VS. AGED CATTLE

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Roles and Contributions of Co-authors: Fernanda Dias helped in tissue collection, sample processing and setting up RT-qPCR. Marc-Andre helped in data analysis and critically reviewed the manuscript, Gregg Adams helped in tissue collection, study design and critically reviewed the manuscript, and Jaswant Singh supervised, helped in study design, data analysis and critically reviewed the manuscript.

Relationship of this study to the dissertation

In earlier studies of the dissertation, granulosa cells of the dominant follicle at the time of selection and preovulatory stage were analyzed separately to determine the age-associated changes in the transcriptome. In this study, granulosa cells of the dominant follicle at the time of selection were compared to preovulatory follicle in young cows and aged cows to differentiate age-related changes during the follicle growth from those occurring during the normal follicular development. Thus, this study explores stage specific changes in granulosa cells of the dominant follicle during the follicle development to explore the pathways and molecular functions that are compromised due to advanced maternal age in cows.

6.1 Introduction

In mammals, a major outcome of reproductive aging is the decline in fertility (Volarcik *et al.*, 1998; Malhi *et al.*, 2007). The age related decline in fertility is not only attributed to compromise oocyte competence but also to decrease ovarian follicle reserves, and altered endocrine and local follicular conditions (De La Fuente & Eppig, 2001; Malhi *et al.*, 2005; Malhi *et al.*, 2006; Malhi *et al.*, 2007; Ireland *et al.*, 2009; Krisher, 2012) High levels of oxidative phosphorylation, mitochondrial dysfunction and chromosomal abnormalities are postulated as potential reasons for lower fertilizing ability of oocytes from aging women (Volarcik *et al.*, 1998) and cattle (Takeo *et al.*, 2013). Understnading the factors that contribute to the decline in fertility may lead to design of new therapeutic protocols to improve the outcome of in vivo and in vitro fertilization procedures and pregnancy rates in both humans and animals.

A healthy population of granulosa cells is necessary for dominant follicle development, oocyte competence and fertility in cattle (Evans *et al.*, 2008). Granulosa cells are closely associated with the oocyte and proposed as potential biomarkers to predict oocyte quality in mammals (Hamel *et al.*, 2008; Assidi *et al.*, 2011). Autocrine, paracrine, and endocrine functions of granulosa cells change in a phase-specific manner during dominant follicle differentiation (Singh & Adams, 1998; Singh & Adams, 2000; Palma *et al.*, 2012). In cattle, granulosa cells of the dominant follicle at the time of follicle selection express specific genes that are involved in cellular proliferation, differentiation, tissue remodeling, estrogen synthesis and apoptosis (Bao *et al.*, 1997; Evans *et al.*, 2004; Mihm *et al.*, 2008; Liu *et al.*, 2009). Likewise, granulosa cells of the preovulatory follicle express specific genes that regulate ovulation, luteinization and oocyte maturation after LH surge (Gilbert *et al.*, 2011; Gilbert *et al.*, 2012).

Major changes in follicular and endocrine patterns have been documented in the bovine model of reproductive aging (Malhi *et al.*, 2005; Malhi *et al.*, 2006; Malhi *et al.*, 2007; Malhi *et al.*, 2008). These changes parallel the endocrine and ovarian alternations observed in women during the perimenopausal period (Adams *et al.*, 2012). Comparison of gene expression in 15 years old cows with younger cows using bovine-specific microarrays revealed that granulosa cells of older cows at the time of selection had decreased ability to regulate gonadotropins and decreased lipid and cholesterol metabolism, cellular proliferation and intracellular communication in older cows (Khan *et al.*, 2012). Similarly, transcriptome analysis of the granulosa cells of the preovulatory follicle from aged cows revealed a delayed response to LH, decreased differentiation and progesterone synthesis (Chapter 5) compared to young cows. Likewise luteinized granulosa cells in aged women had decreased capacity to produce steroids (Pellicer *et al.*, 1994), glycoproteins (Seifer *et al.*, 1996), anti-oxidative enzymes (Tatone *et al.*, 2006) and had increased mitochondrial damage (Seifer *et al.*, 2002). These findings substantiate the perception that maternal aging modulates the functions of granulosa cells during follicular development.

A dominant follicle after the time of selection undergoes through growing, static and regression phases if the corpus luteum is functional (Ginther *et al.*, 1989) or becomes preovulatory follicle and ovulates if plasma progesterone levels decline. These phases are preceded or accompanied by changes in gene expression of follicular cells. In the past, research has been focused on the genes that are expressed in the bovine granulosa cells of the dominant follicle and undergo down-regulation by hCG to understand the involvement of the genes in follicular growth, luteinization and ovulation (Ndiaye *et al.*, 2005). To understand the effect of LH on preovulatory follicle differentiation, bovine granulosa cells have been analyzed at 2 h

before LH surge, and at 6 h and 22 h after the induction of LH surge (Gilbert *et al.*, 2011).However, the extent to which maternal age can modulate the granulosa cell function and differentiation between the periods of follicle selection and ovulation is not well understood. The objective of the current study was to examine this aspect during which we compared the shift in transcriptome of granulosa cells of dominant follicle from aged versus young cows between the time of follicle selection and the preovulatory stage (24 h post-LH treatment). We tested the hypothesis that the fewer genes will be differentially expressed in the granulosa cells of dominant follicle between the time of selection and the time of ovulation in aged cows than in the young cows.

6.2 Materials and methods

6.2.1 Animals

Hereford-cross beef cattle (aged cows, n=7; young cows, n=6) born on the University of Saskatchewan Goodale cattle Farm were maintained together in a single corral during the study period. Their calving history records were available. They had not been pregnant or lactated in the previous two years. Mean age of aged cow group was 16 ± 1.2 years (mean ± SEM) and that of young age group was 8 ± 0.7 years. Five out of six young cows were daughters of the aged cows. Two aged cow did not had a daughter; one of them was matched with a younger cow from the herd whose age was similar to the remaining duaghters. Mother-daughter pairs (n=5 pairs) were used to collect tissues for microarray experiment (n=3 dominat follicles at the time of selection and n=3 preovulatory follicles per age group); therefore, one mother-daughter pair was used twice to collect both type of follicles (at 5 month interval). Three aged and four young cows from microarrays experiment were also used to collect tissues for real-time quantitative PCR (RT-qPCR). One cow per age group was used twice to collect opposite type of follicles for RT-

qPCR experiment. The experimental protocol was approved by the University of Saskatchewan Committee on Animal Care and procedures were conducted in compliance with the guidelines of the Canadian Council on Animal Care.

6.2.2 Ultrasonography, hormonal treatments and follicular dynamics

For the purpose of granulosa cell collection, ovulation synchronization was performed by given a luteolytic dose of prostaglandin (PGF2α; Lutalyse, 25 mg i.m., Pfizer Animal Health, Kirkland, QC, Canada) and ovaries were examined daily till the day of tissue collection by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SD 900, Tokyo, Japan). Size and location of all follicles >3mm was recorded and an animal was considered to be ovulated when a large follicle disappeared at the next ultrasound examination (Malhi *et al.*, 2005)). The day of ovulation was taken as the day of emergence of the first follicular wave (Day 0) (Singh *et al.*, 1997; Malhi *et al.*, 2005). The largest follicle of a wave was defined as the dominant follicle (Ginther et al., 1989; Jaiswal et al., 2004) and Day 3 was considered as the Day of follicle selection (Ko *et al.*, 1991; Adams *et al.*, 1992). If an animal was assigned to "Dominant follicle at the time of selection" group, follicular contents were collected (see next section) on Day 3. To collect tissues from the preovulatory follicles, cows were given PGF2α i.m. on Day 4.5 and 5 (to induce luteolysis), LH (Lutropin, 25 mg i.m., Bioniche Animal Health, Belleville, ON, Canada) on Day 6, and granulosa cells were harvested on Day 7. Follicular data (diameters of dominant and largest subordinate follicles, growth rates) were compared between the aged and young cows (n=6 records per group).

6.2.3 Tissue collection for micorarrays and RT-qPCR

Antral Granulosa cells were collected at two stages of follicle development (dominant follicle at the time of selection and preovulatory dominant follicle 60 h after luteolysis and 24 h

after LH) from aged and young cows either by ultrasound-guided follicle aspirations or after surgical removal of ovary containing the dominant follicle. A total of 12 follicles from mother-daughter pairs (n=5 pairs) were used to collect cell samples for microarray analysis (3 dominant follicles at the time of selection and 3 preovulatory follicles from aged and young cows). All follicles originated from a different animal except one mother-daughter pair (from which the dominant follicles at selection were obtained first) was used again after a 5-month interval to collect the preovulatory follicles. All follicles were collected by ultrasound-guided follicle aspirations except three dominant follicles at the time of selection (one in aged cows and two in young cows).

Likewise, antral granulosa cells were collected from another set of 12 follicles for RT-qPCR analysis (3 dominat follicles at the time of selection and 3 preovulatory follicles from aged and young cows). No follicles were shared between the microarray and RT-qPCR analyses. Due to limited number of animals available, 3 aged and 4 young cows from the microarray tissue collection group were used second time to obtain the opposite type of follicle for RT-qPCR. In addition, one cow per age group was used twice to collect opposite type of follicles for RT-qPCR experiment.

Ultrasound-guided follicle aspirations were performed by transvaginal approach (Singh & Adams, 1998; Dias *et al.*, 2013); using a 5 MHz convex-array transducer (Aloka SSD 900, Tokyo, Japan). A disposable needle (catalog # 305833, 18 ga x 1.5”; BD Medical, Mississauga, ON, Canada) connected to a silicone tubing (115 cm long, 1.14 mm internal diameter, Cole-Palmer, Montreal, QC, Canada) and a 10 ml syringe was used to puncture the dominant follicles and to aspirate the follicular contents as described (Berfelt *et al.*, 1994). Caudal epidural anesthesia was induced with 2% lidocaine HCl (catalog #. 1LID009P; Bimeda-MTC Animal

Health Inc., Cambridge, ON, Canada) and the ovary was positioned against the transvaginal transducer face by transrectal manipulation. The cumulus-oocyte-complex (COC) was separated from the aspirate under a stereomicroscope and antral granulosa cell pellet was obtained by centrifugation of the remaining follicular contents at 700g for 15 minutes. Follicular fluid was stored at -80 °C. Granulosa cells were either snap frozen in liquid nitrogen and stored at -80 °C or suspended in 300-700 µl of RNA stabilization and protection solution (RNA*later*, catalog #. AM7020; Life Technologies, Burlington, ON, Canada) for 12 h at 4 °C before storing at -80 °C.

In order to obtain a greater number of antral granulosa cells, unilateral ovariectomies were performed by colpotomy (Singh *et al.*, 1998) as described. Briefly, caudal epidural anesthesia was induced as described above, an incision was made in the dorsolateral part of the vaginal fornix and the peritoneum was ruptured manually. Local anesthesia was applied to ovarian pedicle and attachments cut by slowly tightening the chain of ecraseur (Jorgensen Labs, catalog #. J0037E, Loveland, Colorado, USA). The cows were treated daily for four days after surgery with procaine penicillin G (Pen G injection; 21000 IU/Kg, i.m., Citadel Animal Health, Edmonton, AB, Canada). The size of the dominant follicle in the excised ovary were measured and confirmed with ultrasound records. A 20 gauge needle attached with 3ml syringe was used to aspirate the follicular contents, syringe was deattached from the needle and the needle was left in place. Collapsed follicle was flushed 3-times with Delbecco's phosphate buffer saline (DPBS, catalog #. 21600-010; Life Technologies, Burlington, ON, Canada). The cumulus-oocyte-complex (COC) was separated from the aspirate and discarded. Content of the first syringe was centrifuged at 700g for 5 minutes to harvest follicular fluid. The cell pellet was mixed with granulosa cells from DPBS contents and centrifuged at 700g for 15 minutes at 4 °C. Follicular

fluid and granulosa cells were processed and stored as described above for ultrasound-guided follicle aspiration procedure.

6.2.4 Follicular fluid analysis for estradiol and progesterone

Follicular fluid of the dominant follicles (n=23 in total; n=5 and n=6 dominant follicles at selection from aged and young cows, respectively, and n=6 preovulatory follicles per age group) were analyzed for estradiol 17β and progesterone concentrations using radioimmunoassay as described (Singh et al., 1998). Briefly, standards and sample dilutions were prepared in charcoal-extracted bovine follicular fluid. The dilution ratio (v:v) for estradiol 17β samples was 1:100 or 1:500 whereas the dilution ratio (v:v) for progesterone samples was 1:40. Radioimmunoassay kits (# KE2D1, Coat-A-Count, Siemens Health Care Diagnostics Inc., Malvern, PA, USA) were used for estradiol 17β and progesterone (# TKOP1, Coat-A-Count, Siemens Health Care Diagnostics Inc.) assays. Sensitivity of estradiol assay ranged from 5 to 500 pg/ml whereas the sensitivity of progesterone assay ranged from 0.1 to 40 ng/ml. All samples were processed as duplicates in the single run. Intra-assay coefficients of variations for estradiol and progesterone assays were 11% and 2.5%, respectively.

6.2.5 RNA extraction, amplification and labeling

RNA extraction, amplification and labeling of granulosa cells was done as described previously (Dias et al., 2013). Briefly, total RNA of granulosa cells was extracted (RNA Isolation Kit # KIT0204; Life Technologies, Burlington, ON, Canada) and eluted in 15 µl of the buffer as prescribed in the manufacturer's protocol. Quality and quantity of RNA was determined using bioanalyzer (Model # 2100; Agilent Technologies, Agilent Technologies, Santa Clara, CA, USA). For microarray analysis, total RNA (5 ng) was linear amplified (RNA amplification Kit # KIT0525; Life Technologies, Burlington, ON, Canada) and 2 µg of

antisense-RNA was labeled with Cy3 and Cy5 florescent dyes (ULS florescent labeling kit # EA-021; Keratech Biotechnology, Amsterdam, The Netherlands) according to the manufacturer's protocol. Efficiency of the both labeling dyes was measured (NanoDrop Technologies; Wilmington, DE, USA) and the non-reacted residual labeling dye was filtered out (DNAse treatment was omitted; RNA isolation kit # KIT0204; Life Technologies, Burlington, ON, Canada).

6.2.6 Microarray design, hybridization, and scanning

Each custom-built bovine oligo-array slide used for this study consisted of 4-arrays. Each microarray contained 37,351 targets (genes and isoforms) excluding the controls in 44x44K format (EmbryoGENE EMBV3, Agilent Technologies) (Robert et al., 2011). Experiment was set up to make direct comparisons between the two stages of follicle development. In a biological replicate, 825 ng of Cy3-labeled antisense-RNA (aRNA) from a dominant follicle at the time of selection was hybridized with 825 ng of Cy5-labeled aRNA of prevoulatory follicle on an array (n=3 comparisons per age group). In addition, the same two follicles were compared after swapping the dye colors (i.e., Cy5 for dominant follicle at selection versus Cy3 for preovulatory follicle) to control non-biological variation (Robert et al., 2011). Thus, six hybridizations (three biological and three technical replicates) were performed per age group by incubating the microarray slides were at 65 ºC for 17 h in a rotating oven @ 10 rpm. All the slides were washed, dried and scanned by a Power Scanner (Tecan Group Ltd. Mannedorf, Switzerland) for image acquisition as described previously (Dias et al., 2013). Signal intensities for individual targets were recorded using Array-Pro software (Media Cybernetics, Inc., Rockville, MD, USA).

6.2.7 Data normalization and statistical analysis

Microarrays signal intensities were processed and statistically analyzed as described previously (Dias et al., 2013). Linearity, specificity, and variability of hybridization were assessed using MIAME-compliant platform of EmbryoGENE laboratory information management system and the microarrays analysis (http://elma.embryogene.ca). Median signal intensities data files were normalized within and between arrays, and statistically analyzed to produce a list of differentially expressed genes using Flexarray software (version1.6.1) as described previously (Michal Blazejczyk, 2007). A gene was considered to be differentially expressed if there was ≥ 2-fold change in its expression with P-value of ≤ 0.05. Data were deposited in NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) with a GEOseries accession number of (This will be acquired once the manuscript is ready for submission).

6.2.8 Functional analyses

Differentially expressed genes (≥ 2-fold change; P ≤ 0.05) in granulosa cells of the preovulatory follicle vs. dominant follicle at selection phase were processed by pathway analysis software (Ingenuity Pathway Analysis; www.ingenuity.com) to understand the biological context (functional annotation, pathways, networks and upstream regulators). To highlight the age associated differences in the transcriptome of granulosa cell during follicular development, comparative analysis of the dataset from aged cows was done with similar dataset from young cows. Average linkage clustering method (cluster 3.0) based on the pathway scores obtained from Ingenuity software was used to produce a heat map (Java Treeview). These heat maps allowed the comparison of canonical pathways between aged vs. young cows during follicular development.

6.2.9 Real time quantitative polymerase chain reaction (RT-qPCR)

Real-time qPCR analyses were performed for a total of six genes from aged cows samples and eight genes from young cow samples (n=3 samples per follicle type). Relative amounts of mRNA of 5 genes were measured for all follicles in both age groups: Tribbles (TRIB2); Inhibin beta A (INHBA), Low density lipoprotein receptor related protein-8 (LRP8), Serine peptidase inhibitor-clade E2 (SERPINE2) and Vanin (VNN1). In addition, expression of three genes (proliferating cell nuclear antigen (PCNA), Nuclear receptor family 5 subtype A2 (NR5A2), Growth arrest and DNA damage 45 subtype B (GADD45B) were measured only in young cow group. Four housekeeping genes (Ubiquitin-conjugating enzyme-E2D2 (UBE2D2), Eukaryotic translation initiation factor-2B2 (EIF2B2), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Splicing factor-3a, subunit-1 (SF3A1) were used as reference genes. Forward and reverse primers for the genes of interest and housekeeping genes are listed in Table 6.1

Total RNA (50 ng) was converted into complimentary-DNA (cDNA) using q-Script (catalog # 95047-100; Quanta Biosciences Inc., Gaithersburg, MD, USA). Primer pairs of each genes were designed (Primer 3; http://frodo.wi.mit.edu/primer3/), analyzed for compatibility (OligoAnalyzer 3.1; Integrated DNA Technologies), amplification efficiency (MxPro software; www.genomics.agilent.com; Table 6.1), and specificity (Basic Local Alignment Search Tool, BLAST; www.blast.ncbi.nlm.nih.gov/). After determining the size of PCR product of each gene by gel-electrophoresis, PCR product was eluted (QIAquick gel extraction kit, catalog # 28704; Qiagen Toronto, Ontario) and sequenced (ABI 3730 XL DNA analyzer; Applied Biosystems CA, USA) to determine the quality (Gap 4.4 software; Staden-Package; http://www2.mrc-

lmb.cam.ac.uk/) and specificity (BLAST). Standard curve (10^{-2} to 10^{-9} ng/µl) was prepared from the PCR product of each gene to calculate relative amounts of cDNA in the samples.

Each PCR reaction (25 µl) was comprised of 2 µl of cDNA of each sample, 12.5 µl of SYBR green master mix II (catalog # 600828Agilent Technologies, Burlington, ON, Canada), 1875 nM of each forward and reverse primer, 0.375 µl of reference dye and 6 µl of nuclease-free water. Each sample was run as a single reaction whereas the standard curve of each gene and no template controls (NTC) were run as multiple reactions. The relative amount of each gene was normalized using the geometric mean of four reference genes (UBE2D2, EIF2B2, GAPDH and SF3A1) as previously described (Khan et al., 2013). The normalized relative amount of each gene was compared statistically among follicles using REST software (Qiagen 2009) (Pfaffl et al., 2002).

Table 6.1 Details about the genes and primers used for the validation of microarrays.

Gene	Accession Number	Oligo	Primer pair Sequence (5' to 3')	Amplicon size (bp)	Efficiency (%)
Target Genes					
TRIB2	NM_178317.3	Forward Reverse	CAGACCTTGGATGCTCTATTCC CAGGGAAGAAAAACAGGTCCTT	202	108.2
LRP8	NM_001097565.1	Forward Reverse	ACGCAAAGTTCTCGCAAGCTCA TGCCATTTCCTCCTCAAACAGG	446	117.9
SERPINE2	NM_174669.2	Forward Reverse	ATCTTGCATTACTTTGGGGGTA AGACCAGTAGTTGACAGGCACA	168	117.2
PCNA	NM_001034494.1	Forward Reverse	TCTCAGTCACATTGGAGATGCT TAGGAGACAGTGGAGTGGCTTT	221	101.4
NR5A2	NM_001206816.1	Forward Reverse	AACAGAAAAAGAACACGGAAG CTACTGGGGAAGATTTGAAGCAC	157	107.3
GADD45B	NM_001040604.1	Forward Reverse	CCAGGACAGTACTTTGGGACTT ATCTGTAAGCTTCCCCTCTGTC	150	108.0
INHBA	NM_174363.2	Forward Reverse	CCAAAGGATGTACCCAACTCTC GTCCGATGTCGTCCTCTATCTC	196	96.5
VNN1	NM_001024556.2	Forward Reverse	TATTCTCTTCCACGATCCTGCT TTCCACTCCCTGTCATTTTCTT	197	105.3
TNFAIP6	NM_001007813.2	Forward Reverse	AAGGAGTGTGGTGGTGTGTTTA TCAACATAGTCAGCCAAGCAAG	185	103.0
Housekeeping Genes					
EIF2B2	NM_001015593.1	Forward Reverse	CATGAGATGGCAGTCAATTTGT CTTGAACATAGGAGCACAGACG	219	97.3
GAPDH	NM_001034034.1	Forward Reverse	CCAACGTGTCTGTTGTGGATCTGA GAGCTTGACAAAGTGGTCGTTGAG	275	99.0
SF3A1	NM_001081510.1	Forward Reverse	TGTGTCCCTCTTGCTGAGTTT ATTCCTGGTTTCACGTCTCCTA	194	96.6
UBE2D2	NM_001046496.1	Forward Reverse	TGGACTCAGAAGTATGCGATGT CTTCTCTGCTAGGAGGCAATGT	242	102.8

6.3 Results

6.3.1 Follicular dynamics and hormonal concentrations

Within aged and young cow age groups, the diameters (mean ± SEM) of the dominant follicle at selection (Day 3 of wave) and the preovulatory follicle (Day 7 of wave; 24 hr after exogenous LH) differed ($P < 0.01$) from their corresponding first subordinate follicles (Table 6.2). Daily growth rate of the dominant follicles between Day 3 and Day 7 of follicular wave were similar (P=0.62) between the aged and young cows (1.5 ± 0.4 and 1.3 ± 0.3), respectively).

In aged and young cows, intrafollicular concentration of estradiol 17β (ng/ml) tended to differ (P=1.0) between the dominant follicle at selection and the preovulatory follicle within as well as between the age groups (Fig. 6.1). In both aged and young cows, intrafollicular progesterone concentrations (ng/ml) were higher ($P < 0.01$) in the preovulatory follicle as compared to dominant follicle at the selection. Estradiol 17β to progesterone ratio differed ($P < 0.01$) in dominant follicles at selection in aged cows (3.0 ± 0.3) vs. young cows (4.9 ± 0.4) but similar (P=0.21) between the two age groups in preovulatory follicle (aged cows 0.8 ± 0.3 vs. young cows 0. 4 ± 0.1).

6.3.2 Differential gene expression

Gene expression data (preovulatory follicle vs. dominant follicle at selection) of young cows was used as reference. In young and aged cows, a total of 2260 and 1209 genes or isoforms (respectively) were differentially expressed at threshold fold change ≥ 2 ($P \leq 0.05$) in granulosa cells of the preovulatory follicle compared to the dominant follicle at selection (Fig. 6.2A). The proportion of the down-regulated transcripts was higher (Chi Square $P < 0.01$) in young cows (56%) compared to aged cows (36%). Up- and down-regulated transcripts from these two gene

lists were compared between the aged and the young cows. A total of 334 up-regulated and 99 down-regulated transcripts were unique to the aged cows and a total of 503 up-regulated and 821 down regulated transcripts were unique in the young cows (Fig. 6.2B). Top ten up and down-regulated genes having largest fold change in their expression in aged and young cows are shown in Table 6.3. None of the top 10 up-regulated genes is the same while three genes are similar in the down-regulated gene lists between the two age groups.

Table 6.2 Comparison of diameters (Mean ± SEM) of dominant follicles between aged and young cows.

	(Mean ± SEM)	
Follicle Diameters	**Aged cows (n=6)**	**Young cows (n=6)**
Dominant follicle at selection	9.5 ± 0.6[a]	8.8 ± 0.3[a]
1st Largest subordinate follicle	7.3 ± 0.2[b]	7.1 ± 0.4[b]
Preovulatory follicle 24 h after LH	15.5 ± 0.4[a]	14.1 ± 0.9[a]
1st Largest Subordinate follicle	6.1 ± 0.5[b]	6.1 ± 0.7[b]
Follicle Growth rates		
Day 0 to Day 3		
Dominant follicle at selectio	1.5 ± 0.1[a]	1.4 ± 0.1[a]
Preovulatory follicle	1.6 ± 0.2[a]	1.8 ± 0.2[a]
Day 3 to Day 7		
Preovulatory follicle	1.5 ± 0.4[a]	1.3 ± 0.3[a]

[a, b] Denote differences between the age groups for dominant follicles ($P < 0.05$) as well as for respective dominant follicle and 1st largest subordinate follicle within each aged group.

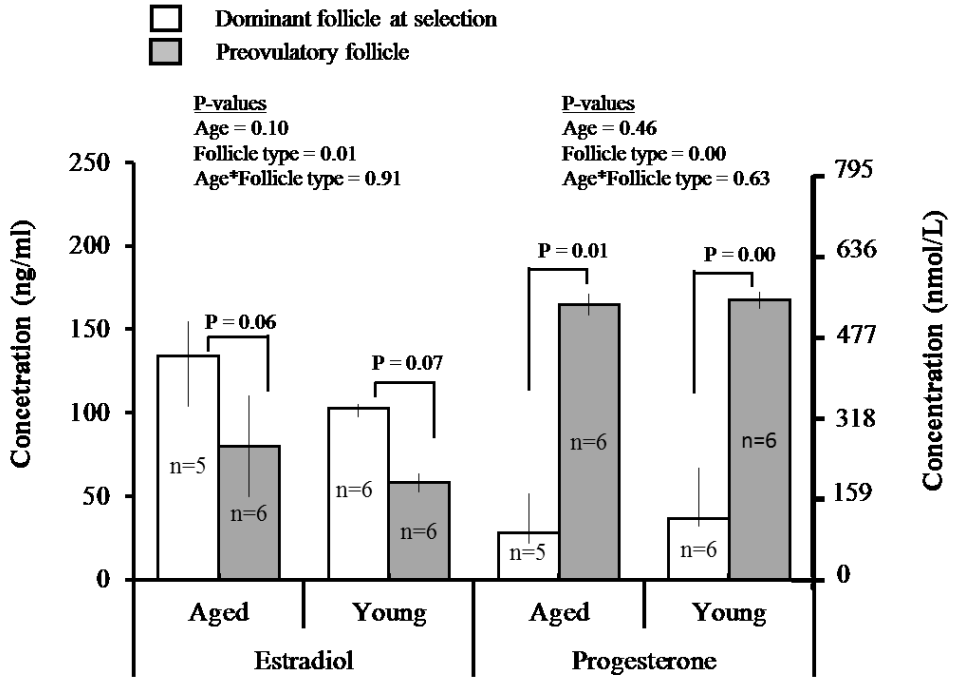

Figure 6.1 Comparison of intra-follicular concentrations (ng/ml; primary vertical axis and nmol/L; secondary vertical axis) of estradiol 17β and progesterone between preovulatory follicle 24 h after LH treatment and dominant follicle at selection from aged (n=5 for dominant follicle; n= 6 for preovulatory follicle) and young (n=6 for dominant follicle; n=6 for preovulatory follicle) cows. Estradiol 17β concentrations tended to be higher at dominant follicle stage compared to preovulatory follicle within the age groups. Also Estradiol 17β concentrations tended to be different between the two age groups at both stages of dominant follicles. Progesterone concentrations dincreased after treatment with LH.Labels inside the bar represent the number of follicles analyzed for estradiol and progesterone.

Table 6.3 List of top ten up- and down-regulated genes in granulosa cells of preovulatory follicle (compared to dominant follicle at selection) in aged and young cows (≥ 2-fold change in expression; $P \leq 0.05$), as assessed by microarrays.

Gene symbol	Protein encoded	Fold change
Up-regulated in aged cows		
CLDN11	Claudin 11	7.7
TNFAIP6	Tumor necrosis factor alpha induced protein 6	6.2
GFRA1	GDNF family receptor alpha 1	5.9
PTX3	Pentraxtin 3, long	5.4
ASB9	Ankyrin repeat and SOCS box containing 9	5.4
IGF2	Insulin-like growth factor 2 (somatomedin A)	5.2
GFPT2	Glutamine-Fructose-6-Phosphate Transaminase 2	5.0
RGS2	Regulator of G-protein signaling 2, 24kDa	4.8
TM4SF1	Transmembrane 4L six family member 1	4.6
CRISPLD2	Cysteine-rich secretory protein LCCL domain containing 2	4.2
Up-regulated in young cows		
VNN1	Vanin 1 or Vascular non-inflammatory molecule 1	6.9
VNN2	Vanin 2 or Vascular non-inflammatory molecule 2	6.4
TFF3	Trefoil factor 3	6.2
TRIB1	Tribbles homolog 1	6.0
NTS	Neurotensin	6.0
KRT19	Keratin 19	6.0
ANXA1	Annexin A1	5.9
FN1	Fibronectin 1	5.8
LTF	Lactotransferrin	5.6
KRT8	Keratin 8	5.5
Down-regulated in aged cows		
TRIB2	Tibbles 2 homolog 2	-4.2
CALB2	Calbindin 2	-3.2
LRP8	Low density lipoprotein receptor-related protein 8,	-3.0
INHBA	Inhibin, beta A	-2.9
PDSS1	Prenyl (decaprenyl) diphosphate synthase, subunit 1	-2.9
SLC44A3	Solute carrier family 44, member 3	-2.8
ETNK2	Ethanolamine kinase 2	-2.7
SORBS2	Sorbin and SH3 domain containing 2	-2.7
INHBB	Inhibin, beta B	-2.6
FLVCR2	Feline leukemia virus subgroup C cellular receptor family 2	-2.6
Down-regulated in young cows		
TRIB2	Tibbles 2 homolog 2	-5.0
LRP8	Low density lipoprotein receptor-related protein 8	-5.4
INHBA	Inhibin, beta A	-4.9
GJA1	Gap junction protein, alpha 1, 43kDa	-5.0
SRGN	Serglycin	-4.9
CYP19A1	Cytochrome P450, family 19A, polypeptide1	-4.8
SEMA6D	Semaphorin 6D	-4.7
SERPINE2	Serpin E2	-4.3
GRB14	Growth factor receptor-bound protein 14	-4.6
FST	Follistatin	-4.2

6.3.3 Functional classification of transcripts

Molecular and cellular functions: Differentially expressed transcripts in granulosa cells of preovulatory follicles from aged cows (n=1209) and young cows (n=2260) were annotated to molecular and cellular function (Table 6.4). Comparison of the molecular and cellular functions between aged and young cows based on the difference of *P*-value (calculated by ingenuity) for a given process revealed the extent to which age affected the association of the transcripts with key functions ($P < 0.05$, Fig. 6.3). In addition, granulosa cell transcripts related to the 1) energy production, 2) gene expression and 3) nucleic acid metabolism were identified as differentially associated in dataset from young cows but not in aged cows (Fig. 6.3). Granulosa cells transcripts related to 1) cell signaling, 2) protein degradation and 3) free radical scavenging were identified as differentially associatedin dataset from aged cows but not in young cows (Fig. 6.3).

Canonical pathways: Key canonical pathways that were annotated in preovulatory follicles of aged cows (compared to dominant follicle at selection) were: 1) actin nucleation by ARP-WASP complex, 2) regulation of actin-based motility by Rho, and 3) actin cytoskeleton signaling (Table 6.5). Comparison of the top 16 canonical pathways between aged and young cows revealed the extent to which set pathways were affected due to maternal age (Fig. 6.4). Pathways related to 1) mitochondrial dysfunction, 2) protein ubiquitination, 3) GADD45b signaling and 4) cell cycles were differentially associated (higher Z-score) to young cows than aged cows. In contrast, 1) actin related pathways (actin nucleation, actin signaling, acting regulation), 2) integrin signaling, 3) cdc42 signaling, 4) ERK/MAPK signaling, and 5) apoptosis signaling were differentially associated (higher Z-score) to aged cows vs. young cows.

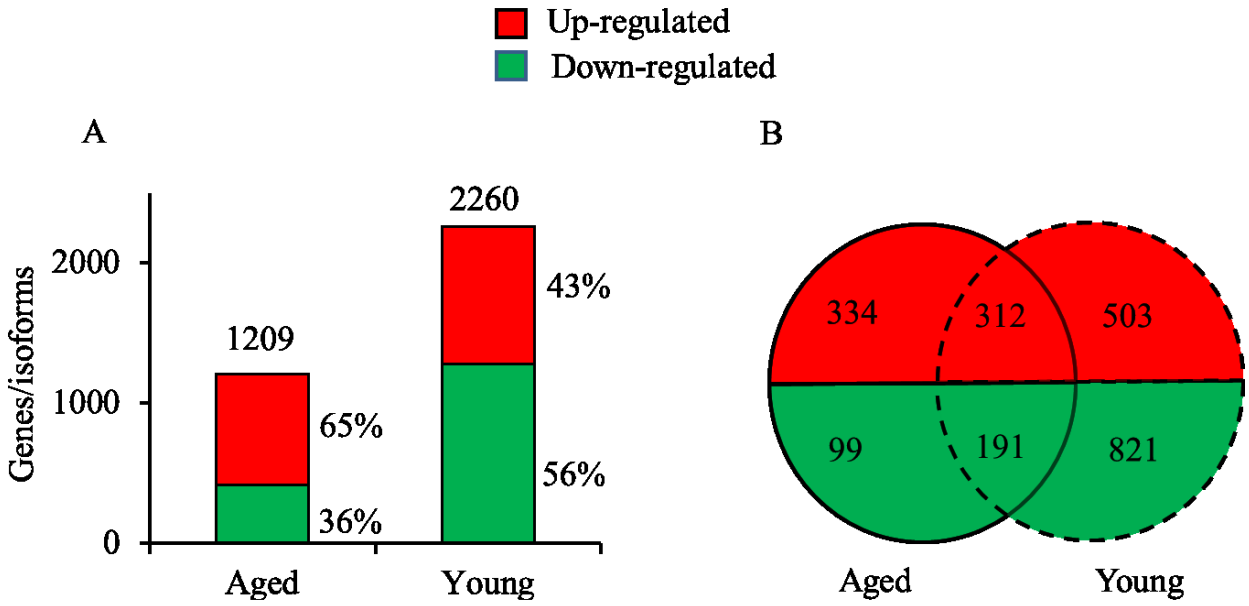

Figure 6.2 Comparison of differentially expressed genes/isoforms in granulosa cells of preovulatory follicle (compared to dominant follicle at selection) in aged and young cows. A) Each stacked bar represents total number of differentially expressed genes or isoforms (threshold $P \leq 0.05$; 2-fold change) as well as up-(red) and down-(green) regulated genes as percentage on right side of each stacked bar at threshold level of 2-fold change ($P \leq 0.05$). B) Venn diagram representing the number of transcripts up-regulated (red, upper) and down-regulated (green, lower) in preovulatory follicles (n=6) compared to dominant follicles at the time of selection (n=6) in aged (solid circle) and young cows (dotted circle; n=5 cows per age group). A total of 312 up-regulated (deep red) and 191 down-regulated (deep green) transcripts were common between the two age groups. The number of genes in Venn diagram for both age groups does not include novel genes.

Table 6.4 List of molecular and cellular functions (in the decreasing order of statistical significance) that were annotated to differentially expressed transcripts in granulosa cells of preovulatory follicles from aged and young cows (compared to dominant follicle at selection). *P*-values indicate a statistically significant (non-random association of the transcripts to a molecular function) and were calculated by the Fisher's Exact Test by IPA software. Transcripts in dataset column list the number of genes in the dataset that were associated with the listed function.

Major Function	***P*-value**	**Transcripts in dataset**
Aged cows (preovulatory follicle vs. dominant follicle at selection)		
Cellular Movement	7.12×10^{-30}	235
Cell Death and Survival	1.22×10^{-28}	330
Cellular Function and Maintenance	1.88×10^{-23}	268
Cellular Development	1.11×10^{-19}	316
Cell-To-Cell Signaling and Interaction	1.22×10^{-13}	171
Cell Morphology	5.06×10^{-13}	214
Cellular Assembly and Organization	2.52×10^{-12}	149
Protein Synthesis	9.13×10^{-12}	126
DNA Replication, Recombination, and Repair	1.87×10^{-08}	57
Cell Cycle	1.15×10^{-07}	119
Cell Signaling	2.31×10^{-07}	56
Lipid Metabolism	4.51×10^{-07}	109
Free Radical Scavenging	3.68×10^{-05}	50
Young cows (preovulatory follicle vs. dominant follicle at selection)		
Cellular Growth and Proliferation	1.77×10^{-26}	522
Cell Death and Survival	1.08×10^{-24}	508
Cell Cycle	6.99×10^{-19}	261
Cellular Development	1.64×10^{-15}	443
Cellular Movement	6.40×10^{-14}	305
Cellular Assembly and Organization	1.36×10^{-13}	195
DNA Replication, Recombination, and Repair	1.36×10^{-13}	197
Cellular Function and Maintenance	2.61×10^{-09}	333
Nucliec Acid Metabolism	1.22×10^{-08}	333
Small Molecule Biochemistry	1.22×10^{-08}	157
Cell Morphology	2.60×10^{-07}	198
Energy Production	4.32×10^{-06}	23

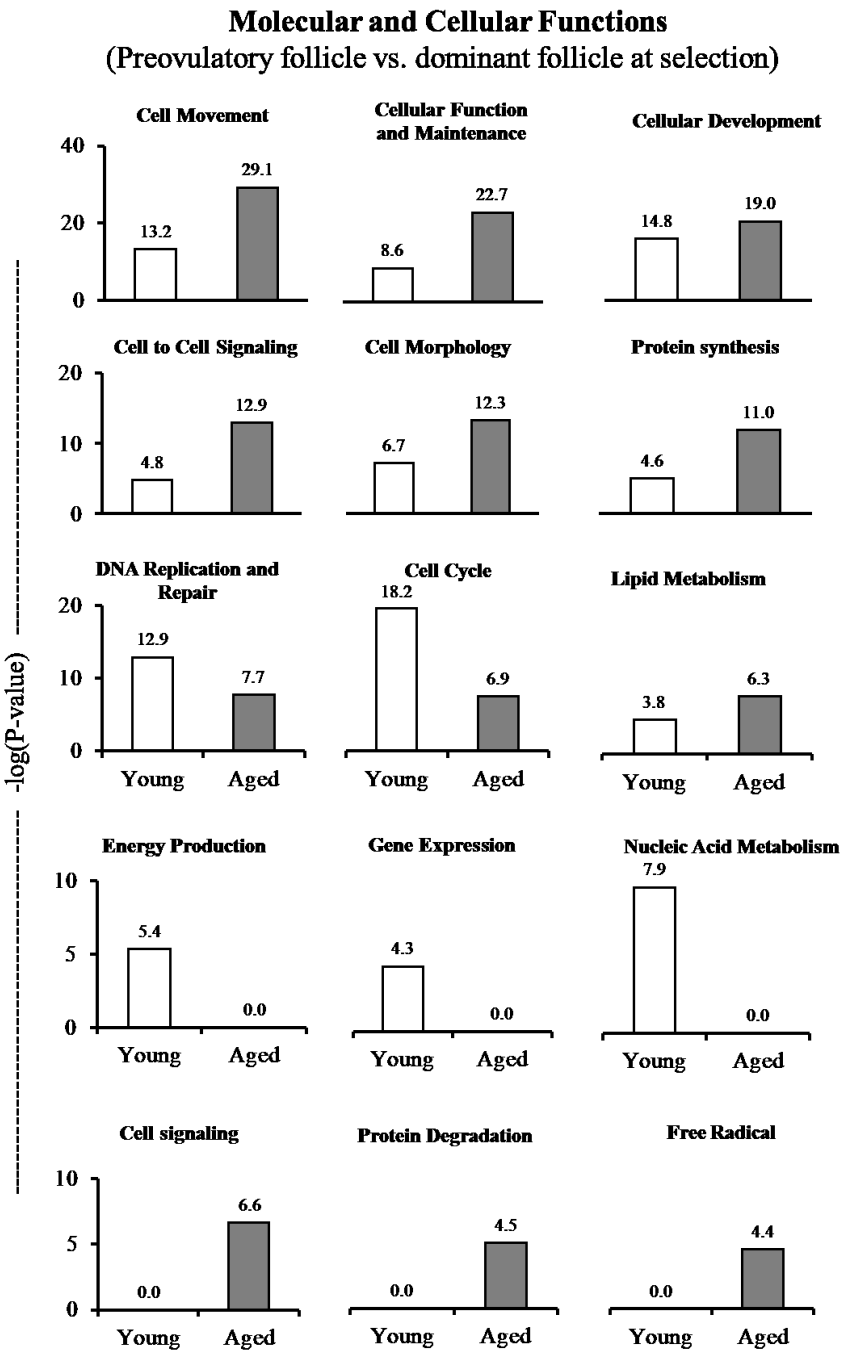

Figure 6.3 Comparison of molecular and cellular functions of granulosa cells between young (open bars) and aged cows (solid bars) for preovulatory follicle (24 h after LH treatment) vs. dominant follicle at selection. Association of transcripts to the specific molecular and cellular functions is affected between aged and young cows. The height of the bar (-log of *P*-value at top of bar) represents the extent by which transcripts in dataset from aged and young cows are associated with a function.

Table 6.5 List of canonical pathways (in the decreasing order of statistical significance) that were annotated to differentially expressed genes in granulosa cells of preovulatory follicle from aged cows (compared to dominant follicle at selection). *P*-values indicate a statistically significant (non-random association of the transcripts to a canonical pathway) and were calculated by the Fisher's Exact Test by IPA software. Transcripts in dataset column are the ratio of the gene list in the dataset over total number of the genes associated with the listed pathway.

Canonical Pathways	***P*-value**	**Transcripts in dataset/total transcripts in Pathway**
Aged (Preovualtory vs. dominant follicle at selection)		
Actin Nucleation by ARP-WASP Complex	8.3×10^{-10}	16/66
ERK/MAPK Signaling	1.8×10^{-07}	26/206
Regulation of Actin-based Motility by Rho	2.5×10^{-07}	16/89
Leukocyte Extravasation Signaling	4.4×10^{-07}	26/197
Actin Cytoskeleton Signaling	6.0×10^{-07}	27/238
Integrin Signaling	9.1×10^{-07}	25/207
CD28 Signaling in T Helper Cells	1.0×10^{-06}	19/132
Phospholipase C Signaling	1.5×10^{-06}	29/260
Cdc42 Signaling	2.1×10^{-05}	18/177
Apoptosis Signaling	2.3×10^{-05}	14/95
Young (Preovualtory vs. dominant follicle at selection)		
Mitochondrial Dysfucntion	3.8 x 10-06	30/177
GADD45 Signaling	4.4 x 10-06	9/22
Protein Ubiquitinition Pathway	1.1 x 10-05	43/268
Mitotic Roles of Plolo-Like Kinase	1.7 x 10-05	16/69
Cell Cycle:G2/M DNA Damage Checkpoint Regulation	7.1 x 10-05	12/48
ATM Signaling	4.8 x 10-04	13/61
Urate Biosynthesis	1.3 x 10-03	7/23
Hereditary Breast Cancer Signaling	1.4 x 10-03	`9/128
Aryl Hydrocarbon Receptor Signaling	1.65 x 10-03	22/161

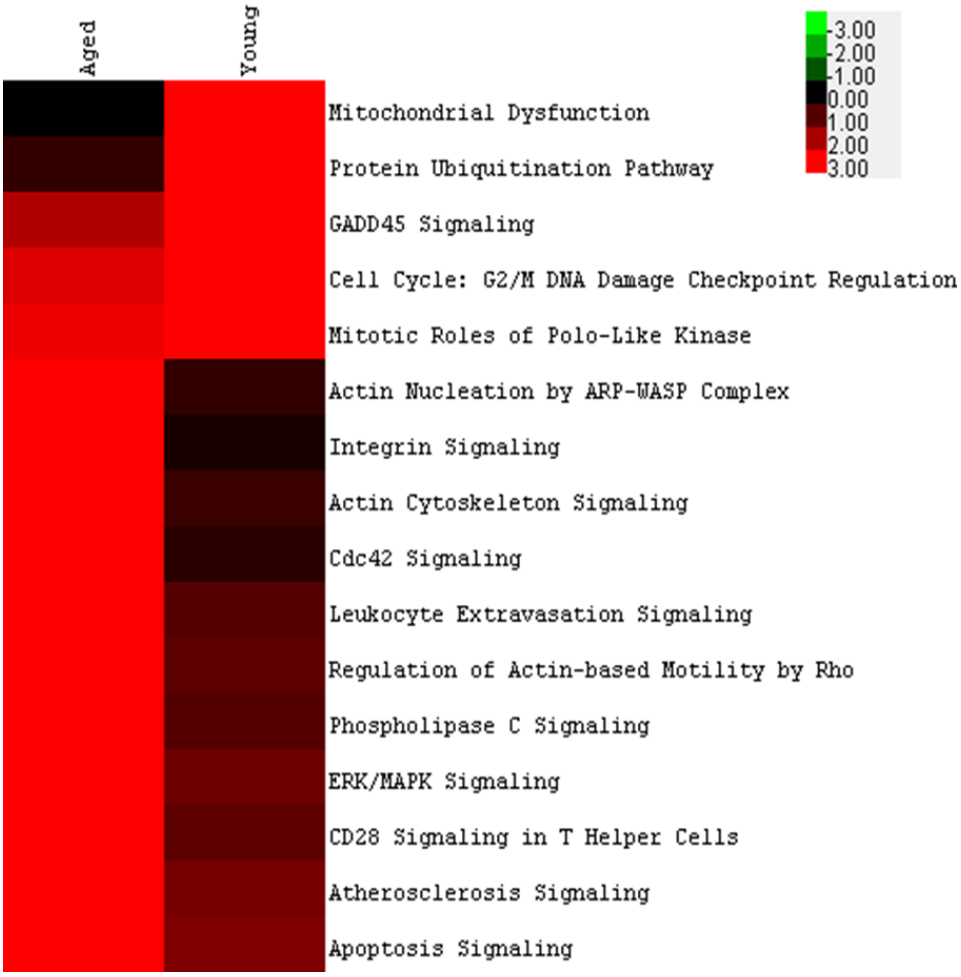

Figure 6.4 Comparison of the top 16 canonical pathways between aged and young cows. Columns represent aged and young cows while rows represent pathways. Pathway score (a measure of differential annontation of a pathway in preovulatory follicles compared to dominant follicles at selection) is represented using a red color gradient (darker = lower score and decreased statistical significance). The score is the negative log of the *P*-value derived from the Fisher's Exact test across all the observations (transcripts) relevant to the pathway using IPA software.

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6.3.4 Network analysis

Inguinity Pathways Analysis software was used to perform network analyses of the differentially expressed transcripts in the preovulatory follicles (compared to the dominant follicle at selection; Section 6.3.6) for aged and young cows (Fig. 6.5). A large number of transcripts related to organization and polymerization of the actin cytoskeleton were upregulated i.e., Actin related protein 3 (ACTR3), Actin related protein complex 2/3, subunit 2 (ARPC2), Actin related protein complex 2/3, subunit 5 (ARPC5), Cortactin (CTTN), Vinculin (VCL), Vasodilator stimulated phosphoprotein (VASP), WASP family protein member 1 (WASF1) in the preovulatory follicles of aged cows but not in young cows (Fig. 6.5A) while mRNA for three genes related to cells differentiation (i.e., Fibroblastic growth factor receptor 2; FGFR2, Early growth response 1; EGR1, Platelet derived growth factor alpha polypeptide; PDGFA) was up-regulated in preovulatory follicles of young cows as compared to the dominant follicle at selection but not in aged cows (Fig. 6.5B). In preovulatory follicles of aged cows, expression of PCNA and cyclin dependent kinase 1; CDK1 (related to growth and proliferation) did not change as compared the dominant follicle at selection but was upregulated in young cows (Fig. 6.5C). Lipid and cholesterol metabolism showed marked differences between aged and young cows (Fig. 6.5D) as several transcripts i.e., Sterol regulatory element binding transcription factor 1 and 2(SREBF1/2), Low density lipoprotein receptor (LDLR), Niemann-Pick disease, type C (NPC), Insulin induced gene 1 (INSIG1), Stearoyl coenzyme desaturase (SCD), HMG-CoA reductase (HMGCR), HMG-CoA synthase (HMGCS1), Cytochrome 450, 51A1, (CYP51A1), ATP citrate lyase (ACLY), and Squalene epioxidase (SQLE) did not expressed in granulosa cells of the preovulatory follicle (compared to the dominant follicle at selection) in aged cows but were down-regualted in the young cows.

6.3.5 Upstream regulators

Inguinity Pathways Analysis software definesan “activated” or “inhibited” upstream regulator by Z-score ≥ 2 or ≤ -2, respectively and predicts if actions of upstream regulators are activated or inhibited based on the expression of the genes in dataset. Key activated upstream regulators involved in regulation of gene expression and pathways in granulosa cells of the preovulatory follicle (compared to the dominant follicle at selection) of aged cows included Tumor necrosis factor (TNF), prostaglandin E2, prostaglandin synthase 2 (PTGS2), CCAAT/Enhancer Binding Protein (C/EBP), alpha (CEBPA), mitogen activated protein kinase 38 (P38 MAPK), ERK, tumor protein 53 (TP53), progesterone receptors (PGR), cAMP responsive element binding protein (CREB1). Likewise, key inhibited upstream regulators included MYC and cytokine molecule CD28. Comparison of upstream molecules between aged and young cows revealed that upstream regulators related to lipid/cholesterol regulation (SREBF1, SREBF2) and cell cycle control (CCND1) were inhibited in the preovulatory follicles (compared to the dominant follicle at selection) in young cows but not in aged cows whereas upstream regulators involved in prostaglandin synthesis (prostaglandin E2 and PTGS2) and lipid metabolism (LDL) were activated in aged cows but not in young cows (Table 6.6).

6.3.6 Validation of differentially expressed transcripts via RT-qPCR

To validate the microarray analysis, mRNA levels of five common transcripts (VNN1, SERPINE2, TRIB2, INHBA and LRP8) between aged and young cows were confirmed by RT-qPCR in granulosa cells of preovulatory follicle relative to the dominant follicle at selection. In both age groups, three independent biological replicates i.e., three aged or young cows per category of follicle (Fig. 6.6A) were used for RT-qPCR analysis. Expression of VNN1 and SERPINE2 in aged cows and mRNA levels of PCNA in young cows were not confirmed by RT-

qPCR analysis; however, expression of these transcripts decreased in both microarrays and RT-qPCR analyses. Expression of two additional genes, i.e., GADD45B ($P < 0.01$), NR5A2 ($P < 0.01$), and was confirmed by RT-qPCR in granulosa cells of preovulatory follicle (compared to dominant follicle at selection) in young cows (Fig. 6.6B).

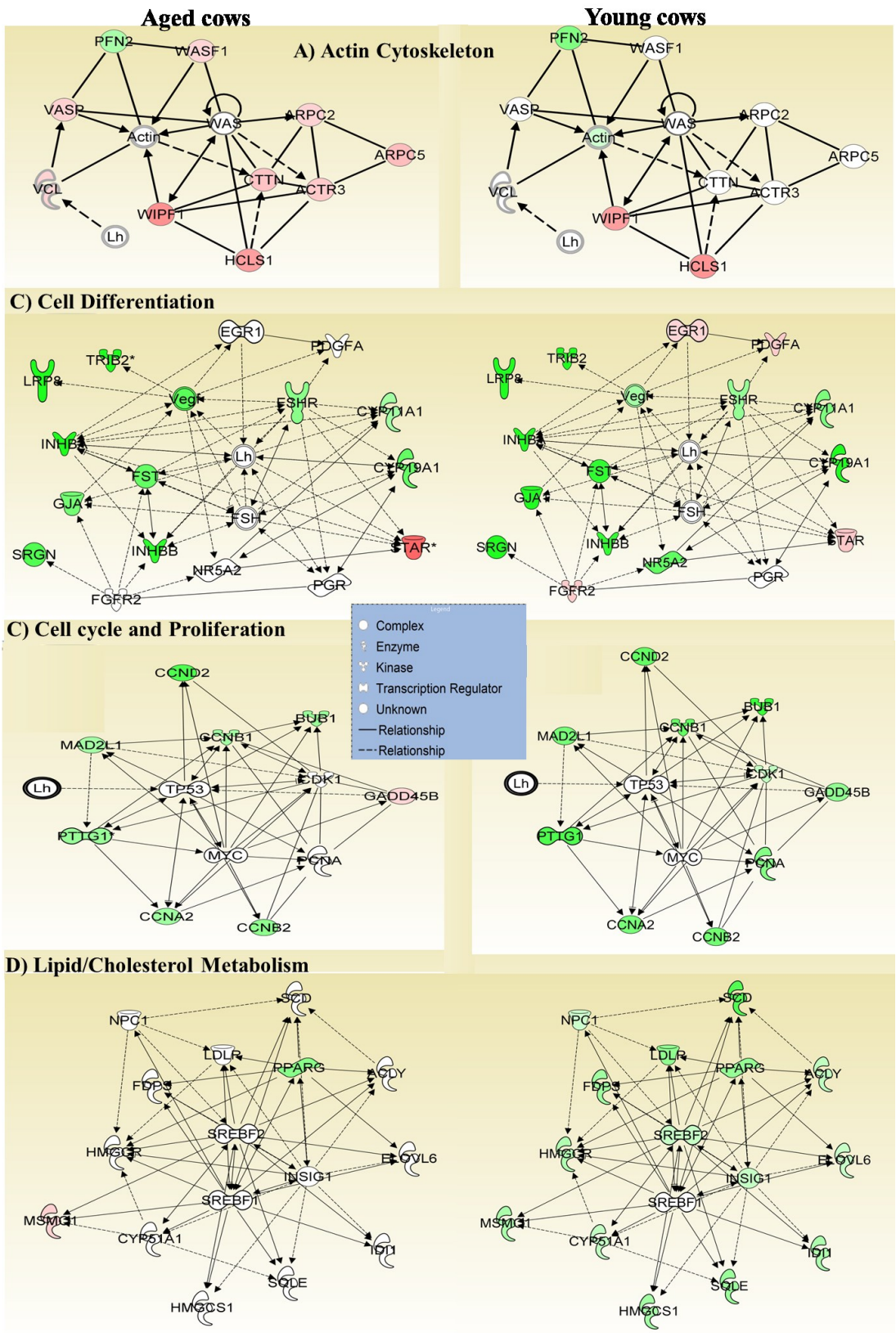
Aged cows
Young cows
A) Actin Cytoskeleton
PFN2
WASF1
VASP
WAS
ARPC2
Actin
ARPC5
CTTN
VCL
ACTR3
WIPF1
Lh
HCLS1
C) Cell Differentiation
EGR1
PDGFA
TRIB2*
LRP8
Vegf
FSHR
CYP11A1
INHBA
Lh
CYP19A1
FST
GJA1
FSH
SRGN
INHBB
STAR*
NR5A2
PGR
FGFR2
Legend
Complex
Enzyme
Kinase
Transcription Regulator
Unknown
Relationship
Relationship
C) Cell cycle and Proliferation
CCND2
BUB1
MAD2L1
CCNB1
Lh
TP53
CDK1
GADD45B
PTTG1
MYC
PCNA
CCNA2
CCNB2
D) Lipid/Cholesterol Metabolism
SCD
NPC1
LDLR
PPARG
FDPS
SREBF2
HMGCR
ELOVL6
INSIG1
SREBF1
MSMO1
CYP51A1
IDI1
SQLE
HMGCS1

Figure 6.5 Network analyses of gene expression in granulosa cells of the preovulatory follicle (compared to the dominant follicle at selection) in aged (left column) and young cows (right column). Each network is comprised of relationships (lines) between genes (nodes) and upstream regulators (central molecules in network) involved in a selected cellular function. Networks representing A) Actin cytoskeleton (upstream regulator– LH), B) Cell differentiation (upstream regulators– LH and FSH, C) Cell cycle and proliferation (upstream regulators– TP53, MYC, and LH) and D) Lipid and cholesterol metabolism (upstream regulators– SREBF1and SREBF2) are illustrated. Color gradient of each node represents up (red) or down-regulation (green) in the expression of the gene and nodes without color represent genes that are not differentially expressed. Illustration generated by Ingenuity Pathway Analysis software.

Table 6.6 Predicted activation status (Z-score; calculated by Fisher's Exact test in IPA software) of upstream regulator molecules (grouped according to molecule type; column 2) for the granulosa cells of preovulatory follicles (compared to the dominant follicle at selection) in aged and young cows. A threshold Z-score ≥ 2 or ≤ -2 was used to detect activation (positive score) or inhibition (negative score) status. A greater absolute value of Z-score denotes high probability of activation or inhibition of upstream regulators.

Upstream Regulator	Molecule Type	Predicted Activation State (Z-score)	
		Aged	**Young**
CEBPA	Transcription regulator	Activated (3.1)	Activated (2.0)
TP53	Transcription regulator	Activated (3.4)	Activated (6.0)
CREB1	Transcription regulator	Activated (3.6)	Activated (2.0)
HIF1A	Transcription regulator	Activated (2.9)	---
PPRC1	Transcription regulator	Activated (2.4)	---
CDKN2A	Transcription regulator	---	Activated (4.1)
MYC	Transcription regulator	Inhibited (-3.2)	Inhibited (-6.8)
PPARGC1A	Transcription regulator	---	Inhibited (-2.5)
PPARGC1B	Transcription regulator	---	Inhibited (-2.1)
GATA4	Transcription regulator	---	Inhibited (-2.3)
SREBF1	Transcription regulator	---	Inhibited (-2.5)
SREBF2	Transcription regulator	---	Inhibited (-2.9)
Prostaglandin E2	Endogenous chemical	Activated (2.9)	---
Cholesterol	Endogenous chemical	---	Activated (3.3)
TNF	Cytokine	Activated (5.0)	Activated (3.3)
SPP1	Cytokine	Activated (2.4)	Activated (2.7)
PI3K	Complex	Activated (2.3)	---
LDL	Complex	Activated (3.1)	---
PKc(s)	Group	Activated (2.3)	Activated (2.3)
ERK	Group	Activated (2.3)	Activated (2.6)
P38 MAPK	Group	Activated (3.5)	---
Rb	Group	---	Activated (2.1)
VEGF	Group	---	Inhibited (-2.1)
CHUK	Kinase	Activated (2.9)	Activated (2.3)
MAPK14	Kinase	Activated (3.7)	Activated (3.7)
RAF1	Kinase	Activated (3.3)	Activated (2.2)
AKT1	Kinase	Activated (2.8)	---
CDKN1A	Kinase	---	Activated (2.3)
PRKAA1	Kinase	Inhibited (-2.2)	---
PRKAA2	Kinase	Inhibited (-2.2)	---
PGR	Ligand-dependent nuclear receptor	Activated (3.2)	Activated (2.6)
IGF1R	Trans-membrane receptor	---	Activated (-2.1)
CXCL12	Other	Activated (4.0)	Activated (2.1)
CD28	Other	Inhibited (-2.0)	---
CCND1	Other	---	Inhibited (-2.0)
CCNK	Other	---	Inhibited (-2.5)
CD24	Other	---	Inhibited (-2.5)
SCAP	Other	---	Inhibited (-2.9)
ANGPT	Growth factor	Activated (3.7)	---
PTGS2	Enzyme	Activated (2.2)	---
PTEN	Phosphatase	---	Activated (4.1)

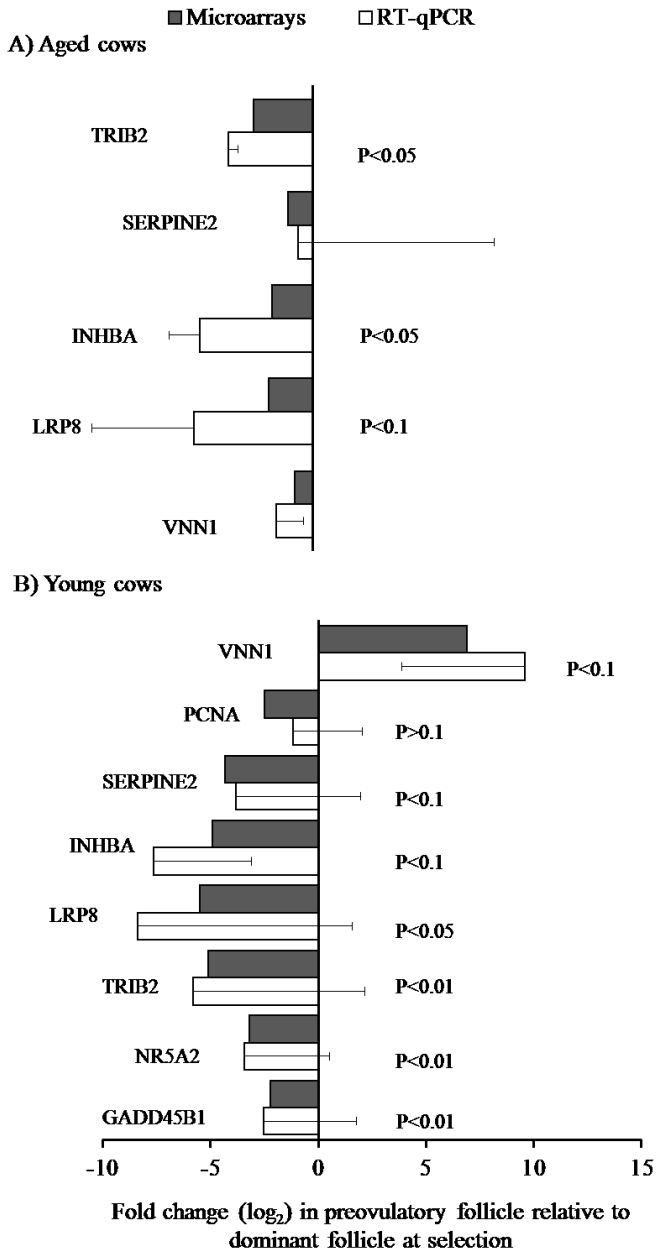

Figure 6.6 Expression of granulosa cell transcripts (log_2 of fold change) by RT-qPCR in preovulatory follicles relative to the dominant follicles at selection in aged cows (A; n=6 follicles in total; 3 follicles per stage from n=5 cows) and young cows (B; n=6 follicles in total; 3 follicles per stage from n=5 cows). Solid bars represent expression of transcripts by microarray analysis. Open bars with standard error (capped line) represent the expression of the same transcripts obtained by RT-qPCR analysis. *P*-value for a statistical difference between preovulatory follicle and dominant follicle at selection for RT-qPCR data is placed to the right of each open bar.

6.4 Discussion

This study examined the effect of maternal age on global gene expression in granulosa cell and dominant follicle function between the time of selection and the prevoulatory stage (24 hr after LH). The hypotheis that fewer genes will be differentially expressed in the granulosa cells of dominant follicle between the time of selection and the time of ovulation in aged cows than in the young cows was supported. The total number of differentially expressed genes or isoforms in aged cows (1206) was 47% less than those of young cows (2206). Only 27% of up- and 17% of down-regulated genes were common between the two age groups. The proportion of the down-regulated transcripts was markedly lower in aged cows (32%) than in young cows (68%) (Fig.6.2). Remarkably, comparison of molecular and cellular functions suggested a weak association of transcripts with functions such as gene expression, nucleic acid metabolism and energy production in aged cows than young cows. Conversely, functions like protein degradation, free radical scavenging and cell signaling were predominantly represented in aged cows than young cows. Table 6.8 summarizes the changes in key functions of granulosa cells of aged and young cows at the time of dominant follicles selection and in the preovualtory follicles 24 h after LH treatment. These findings corroborate the notion that age adversely affects the expression pattern of the genes during dominant follicular development and may be the cause of compromised oocyte competence observed in aged animals.

Compared to the dominant follicle at selection, notable increase in expression and number of granulosa cells transcripts related to the organization and polymerization of actin cytoskeleton was observed in the preovulatory follicles obtained 24 h after LH treatment in aged cows. For example, upregulation of mRNA of actin regulatory proteins ARPC-2 and -5, VCL, VASP and WASF1 was unique to aged cows and was not detected in young cows while mRNA

for WIPFT and HCLS1 were upregulated both in old and young cows. Also, significant association of transcripts with integrins and CDC24 signaling pathways in the granulosa of preovulatory follicles of aged cows suggest involvement of the signaling mechanisms that affect the cytoskeleton. Rearrangement of the actin cytoskeleton is critical for the transduction of endocrine and paracrine steroidogenic signals and coordination of the organelles involved in steroidogenesis process and low level of organization of the actin cytoskeleton is characteristic of steroidogenic phenotype (Sasson et al., 2004). Expression of genes encoding actin cytoskeleton proteins have been shown to be down-regulated after exposure to LH in primary granulosa cell culture in human (Sasson et al., 2004). Concurrent with changes to actin regulation in preovulatory follicles, change in cell morphology and cellular movement functions increased in aged cows. These changes may be related to delayed compensatory changes in response to LH in aged cows reported in a related study (Chapter 5).

Remarkable down-regulation in lipid and cholesterol metabolism happened in granulosa cells of young cows between the time of selection and the time close to ovulation and most of these changes were not detected in aged cows (Fig. 6.5D). Lipid and cholesterol metabolic profile of the preovulatory follicle after LH in aged cows resembled the dominant follicle at the time of selection. It is noteworthy that in the upstream regulators analysis, cholesterol was "active" in young cows but not in aged cows and (Table 6.6). That is, negative feedback effects of cholesterol changed significantly between the time of selection and ovulatory period in young but not in aged cows. Furthermore, transcription regulators SREBF-1 and -2 were down-regulated along with other related transcripts in young cows. These effects may have happened around the time of LH surge but the limitations of the current experimental design do not allow exploring of this notion further. In aged cows, activation of upstream regulator LDL (Table 6.6)

and up-regulation of the transcripts for STAR and others (Fig. 6.5D) in granulosa cells of preovulatory follicles 24 h after LH may be indicative of slower lipid and cholesterol processing (Chaffin et al., 2000). Further, active nature of lipid and cholesterol metabolisam happening at slower place in aged cows is evident by marked inhibition of upstream regulator AMP Kinase alpha subunits 1 and 2 (PRKAA1 and 2), a negative regulator of lipid and cholesterol metabolism (Carling et al., 1987; Tosca et al., 2005), A possible reason of the slower pace of cholesterol metabolism may also be associated with delay in down-regulation of the mRNA transcripts for cytoskeleton regulation (Sasson *et al.*, 2004; Karlsson *et al.*, 2010) in granulosa cells of aged cows. Consequently, both the development of corpus luteum and synthesis of progesterone may be affected in aged cows. This notion is substantiated by the evidence that luteal phase progesterone concentrations were lower, and diameters of corpora lutea are smaller in aged vs. young cows (Malhi et al., 2005).

Peroxisome proliferator activated receptor gamma (PPARG) expression has been reported to be down-regulated after LH surge in rat granulosa cells and plays an important role in steroidogenesis, lipid metabolism, cell cycle, tissue remodeling, angiogenesis and apoptosis (Komar *et al.*, 2001; Komar, 2005). Selective inhibition of upstream molecules PPARGCIA and PPARGC1B (PPARG co-activators) in granulosa cells of preovulatory follicles after LH treatment in young cows (but not in aged cows) indicates the terminal differentiation of follicular cells into luteal cells (Komar et al., 2001). In contrast, upstream regulator PPRC1 (another co-activator of PPARG) was activated (Table 6.6) in granulosa cells of preovulatory follicles of aged cows (compared to dominant follicle at selection); underscoring an activated nature of PPARG in aged cows as compared to young cows. Consequently, follicular preparation for

ovulation and subsequent development of corpus luteum may be slow(Komar, 2005) in aged cows.

Although, differentiation of granulosa cells in aged cows progressed as anticipated by the down-regulation of the transcripts such as FSHR, TRIB2, GJA1, LRP8, Inhibins (beta subunits), SRG, CYP11A1, and CYP19A1 from the time of selection to the time close ot ovulation; however, the degree of down-regulation in terms of fold change was much higher in young cows than aged cows (Fig. 6.5D and 6.8; Table 6.7). Inhibition of upstream molecules IGFR1 in young cows near the time of ovulation (compared to the growing phase at the time of selection) and marked inhibition of protein kinase B (AKT) mediated stimulation of cyclic-AMP response element binding protein (CREB1) indicates follicle growth cessation (Zhou et al., 2013) in young cows. In contrast, upstream regulators, AKT1 and CREB1were markedly activated in aged cows (Table 6.6). Further, the transcripts such as PDGF, EGR1 and NR5A2, that are associated with advanced stage of granulosa cell differentiation (Russell et al., 2003; Saxena et al., 2004; Schmahl et al., 2008), were not expressed in aged cows (Fig. 6.5C). These findings further the hypothesis that despite the ability of the granulosa cells to differentiate in response to LH, gene expression may be affected due to aging (Fig. 6.5B).

Ovulation has been characterized as an inflammatory reaction and involves rapid but transient synthesis of prostaglandins by granulosa cells in the preovulatory follicle after LH surge (Espey, 1980). PTGS2 (Cox2) mediates the synthesis of the prostaglandins in ovarian granulosa cells of rat, cattle and mouse (Sirois *et al.*, 1993; Sirois, 1994). PTGS2 and PGE2 abated by 24 h after LH in granulosa cells of the preovulatory follicle (Monga et al., 2011). In this study, activation of PTGS2 and prostaglandin E2 in the preovulatory follicles 24 h after LH in aged cows shows late induction of inflammatory changes compared to young cows.

Concurrent to the late inflammatory response in aged cows, oxidative stress (increased mRNA of GSTA1, GSTA4 and SOD2) and free radical scavenging were observed in aged cows (Fig.6.3). Therefore, it is tempting to speculate that high levels of oxidative stress in granulosa cells of preovulatory follicle (compared to the dominant follicle at selection) in aged cows may be associated with activation of HIF1A (Kietzmann & Gorlach, 2005) via ERK or P38 MAPK (both were active in aged cows; Table 6.6).

Proliferation of granulosa cells in the preovulatory follicle is transiently halted to achieve maturation of the follicle after LH surge due to increase in levels of cAMP (Richards et al., 2002). In current study, mRNA levels of PCNA, a known marker of proliferation, were not different in aged cows but was down-regualted in young cows when granulosa cells of preovulatory follicle 24 h after LH were compared to the dominant follicle at selection (Fig. 6.5C). The mRNA levels of FOS, an indicator of cellular proliferation, were up-regulated in aged cows (preovulatory vs. dominant follicle) than young cows. In both age groups, the cell cycle genes (CCND2, CCNA2, CCNB2; Fig. 6.5C) were down-regulated and upstream regulator MYC was inhibited (Table 6.6); however, the magnitude of change in gene expression was less severe in aged cows compared to the young cow (Table 6.7). Upstream positive regulators (CCND1, CCNK and CD24) were inhibited while the negative regulators of cell cycle (CDKN2A and CDKN1A) were activated in granulosa cells of preovulatory follicles 24 h after LH (in comparison to dominant follicle at selection) in young cows (Table 6.6). CDKN2A is part of the complex which blocks phosphorylation of retinoblastoma (Rb); thus the cells do not enter S phase of the cell cycle (Agarwal et al., 2012). Similarly, CDKN1A (p21) mediates TP53 (suggested to be activated in young cows) dependent G1 phase cell cycle arrest in response to variety of stimuli. In contrast, there seems to be a delay in achieving transient cell cycle arrest in

granulosa cells of aged cows as only TP53 was activated but others regulators were not inhibited (CCND1,CCNK and CD24) or activated (CDKN1A and CDKN2A). Therefore, these findings lead to hypothesis that granulosa cells from aged cows transiently enter into the cell cycle arrest in response to gonadotropin but the cell cycle regulation is less efficient than in young cows.

To conclude, the hypotheis that fewer genes are differentially expressed in the granulosa cells of dominant follicle between the time of selection and the time of ovulation in aged cows than in the young cows was supported. The proportion of the down-regulated transcripts was markedly lower in aged cows than in young cows. Table 6.8 provides a unique insight about the effect of maternal age on granulosa cell differentiation. Aged cows showed altered rearrangement of cytoplasm and cytoskeleton (↑ARPC2/5, ↑VCL, ↑VASP, ↑WASF1, and ↑VIPF1), inefficient lipid and cholesterol metabolism (↑SERBF 1/2, ↑LDLR, ↑NPC, ↑INSIG1, ↑SCD, ↑HMGCR, ↑HMGCS1, ↑ CYP51A1, ↑ACDY, and ↑SQLE) and differentiation (↓PDGFA, ↓EGR1, ↓NAR5A2), relatively increased proliferation (↑PCNA and inhibition IGF1R) and free radical scavenging (higher oxidative stress; ↑GSTA1, ↑GSTA4 and ↑SOD2), and delayed inflammatory changes (↑PTGS2, activated status of upstream regulator PPARGC). It is likely that these changes in the functions of granulosa cells may lead to inefficient transition of granulosa cells into luteal tissue and may affect the oocyte competence during final stages of dominant follicle development.

Table 6.7 Comparison of magnitude of fold change between aged and young cows. Numbers listed under aged cows and young cows columns represent the gene expression (log_2 fold change at $P < 0.05$ in the preovulatory follicle compared to the dominant follicle at selection. ND denotes the transcripts that were not differentially expressed (at $P < 0.05$ and 2-fold change).

Preovulatory follicle vs. dominant follicle at selection		
Official Gene Symbols	**Log_2 fold change**	
	Aged cows	**Young cows**
Up-regulated		
NTS	3.7	6.0
LTF	2.3	5.6
KRT8	2.8	5.5
ANXA1	3.1	5.9
TRIB1	1.2	6.0
PTX3	2.4	5.5
PLAT	3.8	1.9
STAR	3.7	1.5
RGS2	4.9	2.2
CTGF	ND	2.49
PTGS2	2.3	ND
CDC42	1.1	ND
TNFAIP6	6.2	ND
Down-regulated		
TRIB2	-4.2	-5.0
LRP8	-3.1	-5.4
GJA1	-1.8	-5.0
INHBA	-2.9	-5.0
SRGN	-2.1	-4.9
CYP19A1	-2.0	-4.8
SERPINE2	-1.7	-4.3
FST	-2.0	-4.2
CCND2	-2.2	-3.1
GADD45B	1.1	2.2
NR5A2	ND	-3.2
PCNA	ND	-2.5
CCNA1	-1.2	-2.7
CCNB1	-1.2	-3.2
CCNB2	-1.4	-2.9
CCND2	-2.2	-3.2

Table 6.8 Summary of the changes in key functions of granulosa cells during follicular development. Comparison of granulosa cells of dominant follicle at selection vs. preovulatory stage (24 h after LH treatment) between aged (upper row) and young cows (lower row) indicates increase (upward arrow) or decrease (downward arrow) in the functions (the magnitude of change in the function is shown by number of arrows). Arrows are based on the *P*-value or Z-score for a given function from IPA software.

	Molecualr Functions	**Dominant follicle at selection**	**Preovulatory follicle**
Aged	Cytoskeleton organization	↓↓↓↓	↑↑↑↑
	Organization of cytoplasm	↑↑	↑↑↑↑
	Lipid and cholesterol metabolism	↓↓↓↓	↑↑↑
	Cell cycle and proliferation	↑↑↑	↑↑
	Oxidative stress response	↑↑↑	↑↑↑
	Steroidogenesis	↓↓↓	↑↑
	Cellular Movement		↑↑
Young	Cytoskeleton organization	↓↓	↑↑↑
	Organization of cytoplasm	↑↑↑↑	↑↑↑
	Lipid and cholesterol metabolism	↓↓	↑↑↑↑
	Cell cycle and proliferation	↑↑↑↑	↓↓↓
	Oxidative stress response	↓↓↓	↓↓↓
	Steroidogenesis	↑↑↑↑	↑↑↑↑
	Cellular Movement		↑↑↑

7 CHAPTER 7: GENERAL DISCUSSION

This study encompasses the transcriptome analyses of granulosa cells of bovine dominant follicle to understand the age-associated changes in follicular environment. Follicular development, oocyte competence and development of corpus luteum have been reported to be compromised with advancing age (Volarcik *et al.*, 1998; de Bruin *et al.*, 2004; Malhi *et al.*, 2005). Findings of this thesis provide insight about the transcriptional activity in the granulosa cells and intracellular pathways associated with previously identified changes in the bovine model of reproductive aging (Malhi *et al.*, 2005; Malhi *et al.*, 2006; Malhi *et al.*, 2007; Malhi *et al.*, 2008).

The overall hypothesis of the study was that the transcriptome difference between aged and young cows will be more apparent close to ovulation than at the time of selection of the dominant follicle. In support of this notion, results from the chapters 4 and 5 suggested fewer differentially expressed genes or isoforms at the time of dominant follicle selection between aged and young cows than at the preovulatory stage, i.e., 196 vs. 1340, respectively. Therefore, it is expected that fewer differentially expressed genes in aged cows may not be able to disrupt the selection process of the follicle but will compromise the future development of the dominant follicle. To elucidate it further, differentially expressed gene in aged cows were compared with those from earlier reports, and the results supported the compromised phenotype of the dominant follicle (Table 4.5).

Several studies have documented decreased progesterone concentrations during the luteal phase in aged women (Sherman & Korenman, 1975; Santoro *et al.*, 1996; Hale *et al.*, 2007; Hale & Burger, 2009; Vanden-Brink, 2012) and cattle (Malhi *et al.*, 2005). However, the underlying factors involved in low progesterone synthesis and inefficient development of the

corpus luteum remained unknown. At the time of dominant follicle selection (Chapter 4), transcripts related to the lipid and cholesterol metabolism down-regulated in aged cows compared to the young cows. Concurrently, intrafollicular concentrations of progesterone were lower whereas estradiol concentrations did not differ in dominant follicles in aged cows than young cows (Table 4.2). Likewise, in preovulatory follicles 24 h after LH (Chapter 5), transition from estradiol to progesterone production progressed slowly in aged cows than young cows (Fig. 5.2). Consequently, old cows had increased intrafollicular estradiol and decreased concentrations of progesterone as compared to young and middle-aged cows (Fig. 5.2). Inconsistent lipid and cholesterol metabolism in aged cows were further substantiated by the comparison of the granulosa cells between the time of dominant follicle selection and 24 h after LH treatment in aged and young cows (chapter 6); e.g. transcripts related to lipid metabolism were not differentially expressed in aged cows (Fig. 6.5). One of the possible reasons for decreased progesterone production by the corpus luteum in aged cow (Malhi *et al.*, 2005) could be compromised processing of lipid and cholesterol inherent to the granulosa cells from which the luteal cells originate.

In ruminants and rodents, the cholesterol in granulosa cells is acquired via lipoproteins (LDL and HDL) or by de novo synthesis (Hu *et al.*, 2010). The results of the chapter 4 suggested that the options for cholesterol uptake at the time of dominant follicle selection are limited for aged cows. The uptakes of cholesterol through LDL pathway and de novo cholesterol synthesis were repressed except the HDL-mediated pathway in granulosa cells of aged cows (Fig. 7.1). Consequently, accumulation of cholesterol for the future production of steroids may take longer time in granulosa cells of aged cows. In the current studies, theca cells were not evaluated for cholesterol/lipid metabolism but a compromised lipid and cholesterol metabolism in theca cells

may impair androgen synthesis and result in decrease amount of substrate for estradiol synthesis. In such a scenario, concurrent decrease in aromatase (CYP19A1) from granulosa cells may impair estradiol synthesis at the time of dominant follicle selection. In addition, androgens have been proposed to regulate the ovarian development and functions by preventing the follicular atresia in mouse (Sen & Hammes, 2010). It is reasonable to propose that exogenous androgen administration during the period of active estradiol production in old cows may improve follicle function, oocyte competence and subsequent luteal development. This notion is supported by the results from studies in women with advancing age where dehydroepiandrosterone (DHEA) supplementation increased 1) ovarian response to gonadotropins (increased number of follicles and oocytes) (Barad & Gleicher, 2005), 2) plasma concentration of IGF-1 and high density lipoproteins (Casson *et al.*, 1998), and 3) preganacy rate in women with diminished ovarian reserves (Gleicher & Barad, 2011).

Soon after LH surge or hCG treatment, the follicular cells of preovulatory follicle in women (Veldhuis *et al.*, 1983) and cattle (Fortune & Quirk, 1988) produce increased amount of progesterone. Although the initial rise in progesterone may be due to luteinization of theca cells than granulosa cells (Christenson & Devoto, 2003) yet research indicates that a concurrent luteinization of the granulosa cells is necessary for the progesterone production, ovulation and subsequent development of the corpus luteum (Fortune & Quirk, 1988). In mouse ovulation models, LH or hCG induced a rapid but transient gene expression of steroidogenic enzymes in granulosa cells (Richards *et al.*, 2002; Russell & Robker, 2007). Similarly, gene expression pattern of steroidogenic enzymes (HSD3B and CYP11A1) in granulosa cells of bovine preovulatory decreased by 24 h and later increased by 72 h after the LH surge (Voss & Fortune, 1993). In chapter 5, this transient expression in granulosa cells seems to be delayed in aged cows

as compared to the young cows (Fig. 5.7). This situation may be caused by the delayed synthesis of the mRNA for steroidogenic enzymes or insufficient cholesterol synthesis/uptake in granulosa cell of the aged cows as suggested from the dominant follicle at the time of selection (Fig. 7.1). However, to ascertain the exact reason, study of transcriptome profile of granulosa cells just prior to LH treatment is required.

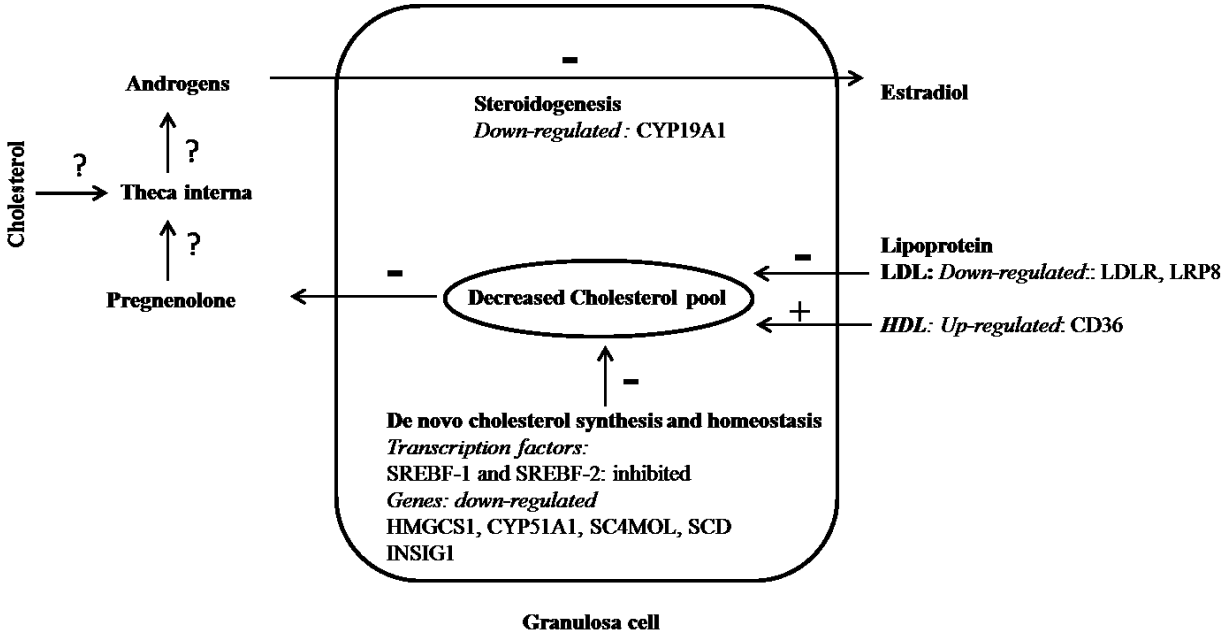

Figure 7.1 Model showing lipid and cholesterol metabolism of the dominant follicle at the time of selection in aged cows. Decreased expression of the transcripts related to LDL and de novo synthesis pathways may lead to sub optimal cholesterol levels in the granulosa cells of aged cows. Consequently, production of pregnenolone and progesterone is reduced from granulosa cells and less amount of pregnenolone is transferred to theca cells for androgen production. Concurrent decrease in aromatase synthesis in granulosa cells and decreased availability of androgen from theca cells may result in decreased estradiol synthesis. Arrows indicate the direction of molecules in or out of the granulosa cell while (-) or (+) signs indicate decreased and increased pace of the process, respectively. (? = unknown).

In chapter 5 of this study, results suggested that the granulosa cells of the preovulatory follicles are responding slowly to ovulatory dose of LH in aged cows as compared to young cows (Fig. 5.7). These results provide an explanation for the delayed ovulation or suboptimal ovulatory response in aged cows and perimenopausal or infertile women (Fitzgerald *et al.*, 1994; McClure N, 1997; Malhi *et al.*, 2008). Late ovulation is associated with either delayed LH surge in aged cows (Malhi *et al.*, 2008) or the decreased magnitude of LH surge in rats (LaPolt & Lu, 2001) due to age-associated changes in hypothalamic-pituitary axis (Nass *et al.*, 1984). However, the role of the granulosa cells in late ovulation has not been investigated in aged cows. Considering that the magnitude of LH did not change in aged cows and delayed LH surge caused late ovulation, we challenged aged cows with exogenous LH to test the preparedness of the granulosa cells 24 h later for ovulation. The results suggested that the transcriptional activity in granulosa cells was slow in aged cows compared with the young cows (Fig. 5.7).

In aged cows, the possible mechanisms involved in delayed ovulation may include: 1) post receptor signal attenuation, 2) delay in rupturing of the follicular wall due to late induction of inflammation and extracellular matrix changes and, 3) decreased or late production of progesterone (Fig. 5.8). The notion of post receptor signal attenuation in granulosa cells of aged cows is supported by increased expression of RGS2 as compared to young cows, despite no difference in mRNA levels of LHR (by microarray expression). RGS2 is involved in desensitization of GPCR by hydrolyzing the GTP to GDP on G-alpha subunit (Sasson *et al.*, 2004) and cause decreased expression of adenylyl cyclase and production of cAMP (Berman & Gilman, 1998; Kehrl & Sinnarajah, 2002) in granulosa cells of aged cows.

The notion of late ovulatory response is supported by increased expression of SERPINE2 in granulosa cells of aged cows. Increased mRNA levels of SERPINE2 prevent the

tissue plasminogen activator to catalyze plasminogen into plasmin (Lu *et al.*, 2013). In turn, plasmins may not actively degrade collagen, fibrin, and laminin in follicular wall. Concurrently, increased mRNA expression of TIMP2 in granulosa cells of aged cows suggests that the activation of matrix metalloproteinases (MMPs) (Liu *et al.*, 2013) may be impeded due to increased mRNA expression of TIMP2 in aged cows (Liu *et al.*, 2013). As a result, the degradation of follicular wall may occur slowly and impede ovulation in aged cows as compared to young cows.

Lastly, the decreased production of progesterone from granulosa cells of aged cows may be responsible for late induction of PTGS2 (increased mRNA levels) and in turn less conversion of arachidonic acid into PGE2 (Bridges *et al.*, 2006; Kim *et al.*, 2009). Consequently, inflammatory process may be slower and expansion of extracellular matrix may also be delayed due to increased mRNA levels of TNFAIP6 by 24 h after LH treatment (Jessen & Odum, 2003). These results suggest a likely delay in follicular maturation and ovulation of the preovulatory follicle in aged cows compared to the young cows. In addition, decreased expression of BTC (EGF-like growth factor) in granulosa cells of aged cows (chapter 5) indicates a likely delay in oocyte maturation. EGF-like growth factors (amphiregulin, epiregulin and betacellulin) are synthesized in granulosa cells and cumulus cells of mammals in response to LH in PGE2 dependent mannar (Freimann *et al.*, 2004; Park *et al.*, 2004; Shimada *et al.*, 2006). These peptides play a role in oocyte meiotic resumption, cumulus cell expansion and ovulation (Shimada *et al.*, 2006).

Age-associated decline in proliferation and DNA damage repair of human fibroblasts and lymphocytes is well documented (Goukassian *et al.*, 2000). In this study (chapter 4), the granulosa cells of dominant follicle at the time of selection possessed decreased mRNA of genes

related to cell cycle and DNA damage repair (Supplementary Table 10.1). The decreased mRNA levels of PCNA in the dominant follicle of aged cows indicated that aged cows had less prolific granulosa cells (Oktay *et al.*, 1995; Tanno *et al.*, 1996) for the development of the ovulatory follicle as compared to young cows. Considering the upstream molecule analysis (Table 4.4) of the study (chapter 4), decreased proliferation in granulosa cells of aged cows may be due to altered response of granulosa cells to plasma gonadotropin (FSH) (Yu *et al.*, 2005), steroids (estrogen) (Evans *et al.*, 2004) and local growth factor (EGF) (Lin *et al.*, 2011) and may also be related to the decreased synthesis of gap junctions (Gittens *et al.*, 2003).

Granulosa cells of the preovulatory follicles have been shown to stop dividing transiently after LH surge to prepare for luteinization and ovulation (Richards et al., 2002). Contrarily, granulosa cells of aged cows showed increased expression of mRNA of PCNA, CCNA2 and transcription factor E2F5 (chapter 5). In addition, upstream regulators such as MYB, MYC were active in granulosa cells 24 h after LH treatment in aged cows compared to the young cows (Table 5.3). Likewise, in chapter 6, mRNA levels of PCNA were not significantly different between granulosa cells after 24 h after LH treatment vs. at the time of the dominant follicle selection in aged cows whereas the same comparison of the granulosa cells in young cows showed decreased mRNA expression of PCNA (Fig. 6.5C). In chapter 6, transcripts related to cell cycle had a similar pattern of changes in mRNA expression (decreased mRNA levels of CCND2, CCNA2, and CCNB2) between the granulosa cells of preovulatory follicle and growing dominant follicle (at selection) in aged and young cows; however, the magnitude of down-regulation of the genes was less evident in aged cows than in young cow (Table 6.7). These findings support the notion that granulosa cells from aged cows actively proliferate 24 h after LH to compensate the slow proliferation at the time of selection of the dominant follicle.

Earlier studies reported higher plasma FSH levels in aged cows throughout the estrous cycle (Malhi *et al.*, 2005). However, the cause of high plasma FSH levels of was not documented. In the current study (chapter 4), decreased mRNA levels of inhibins and follistatin were observed at the time of follicle selection in aged cows as compared to young cows. Inhibins act directly on anterior pituitary to regulate FSH while follistatin suppress FSH via neutralization of activin (de Kretser *et al.*, 2002). These findings support the notion that age-associated increased in plasma FSH may be due to reduced negative feedback of inhibins and follistatin at the pituitary level as reported in aged women (Welt *et al.*, 1999).

Oxidative stress is one of the major causes of maternal aging (Agarwal *et al.*, 2012). Earlier studies have evaluated the impact of oxidative stress in granulosa cells of ovarian follicles (Ito *et al.*, 2008; Liu & Li, 2010; Karuputhula *et al.*, 2013). Antioxidant enzymes such as glutathione peroxidase and superoxide dismutase counter the reactive oxygen species (ROS) to prevent DNA damage (Uttara *et al.*, 2009). In the current study, oxidative stress response was higher at the time of selection of the dominant follicle (chapter 4) as mRNA levels of oxidative response genes such as GPX3, VNN1, and VNN2 increased in granulosa cells of aged cows as compared to young cows. Aged cows at the time of dominant follicle selection showed activation of p38 (MAPK14) and TP53 that caused cell cycle repression and decreased proliferation (decreased mRNA levels of PCNA, PTTG1) to check DNA damage in granulosa cells. In follow up, oxidative stress related pathways such as glutathione metabolism and NRF-2 mediated oxidative stress response were the most significant pathways that were affected by maternal aging at the preovulatory follicle stage in aged cows (chapter 5; Fig. 5.4). In addition, the magnitude of oxidative stress seems to increase with the development of the follicle in aged cows. In support, higher mRNA levels of the GSTA1, GSTA4 and SOD2 and increase in free

radical scavenging (suggested by IPA) were observed at preovulatory follicle stage compared to the time of dominant follicle selection in the aged cows (Fig. 6.3). Altogether, this study provides insight about the involvement of oxidative stress in deteriorating the follicular environment during maternal aging (Liu & Li, 2010).

Cytoskeleton of the eukaryotic cells is composed of microfilaments (actin), intermediate filaments (keratin, vimentin, laminin and microtubule (tubulin) (Wickstead & Gull, 2011). The role of the cytoskeleton has been suggested in the differentiation, cell migration, organelle movement, proliferation and steroidogenesis of the ovarian follicular cells (Sasson *et al.*, 2004). In aged cows, changes in the cytoskeleton of the dominant follicles (chapter 4) were less obvious (decreased mRNA levels of tubulins and increased expression of TPM2; Fig. 4.4) as compared to the young cows. In chapter 6, comparison of the granulosa cells, 24 h after the LH treatment vs. at the time of selection of the dominant follicle indicated that aged cows had increased activity of genes associated with the cytoskeletal components in response to LH compared to the young cows (Fig. 6.5A). Considering these results, we hypothesized that high expression of cytoskeleton genes in the preovulatory follicle of aged cows may be a compensatory response to the slow changes at the time of dominant follicle selection. In conclusion, results suggest that the maternal age alters the cytoskeleton of granulosa cells in the follicle phase-specific manner.

Results from current study (chapter 6) demonstrated that the granulosa cells of the aged cows expressed fewer genes during the follicular development as compared to those from young cows (Fig. 6.2). Cellular functions such as gene expression, nucleic acid metabolism and energy production were increased in young cows compared to the old cows during the follicular development (Fig. 6.3). A likely cause of decreased gene expression in aged cows may be the defective or decreased RNA synthesis as reported in aging eukaryotic cells (Wulff *et al.*, 1962).

Likewise, despite increase demand of energy for DNA damage repair and genomic surveillance, production of energy (ATP) has been reported to be decreased in non-ovarian tissues as part of aging process (Drew *et al.*, 2003; Kirkwood, 2005). Interestingly, we observed that the oxidative phosphorylation and mitochondrial dysfunction pathways were significantly affected in the preovulatory follicle (24 h after LH treatment) of old cows as compared to young cows (Fig. 5.4). These results indicate that the granulosa cells of aged cows may have a decreased capacity for energy production.

Epigenetic mechanisms (DNA methylation, histone modifications) orchestrate cellular homeostasis (Berger *et al.*, 2009). Alterations in epigenetic mechanisms cause functional errors that may lead to altered phenotype and disease (Ben-Avraham *et al.*, 2012). Aging causes modification in epigenome of eukaryotic cells (Ben-Avraham *et al.*, 2012). In current study, we did not analyze data for the gene expression of the chromtin remodeling enzymes such as histone acyltransferases (HATs), histone deacetylases (HDATs) or histone methyltrasnferases (HMTs). Although it is very speculative at this time, these enzymes may silence the gene expression in follicular cells and may be the reason of fewer gene expressions during the follicular development in aged cows.

In the current study, quantitative real-time PCR was used to validate the expression of microarrays. Relative analysis of gene expression by RT-qPCR is done after normalizing the expression of the gene of interest with the expression of reference gene (s) obtained from the same sample. In this context, the selection of stable reference gene is important to quantify the mRNA levels of gene of interest accurately (Haller *et al.*, 2004). Several studies have demonstrated that maternal age influences the expression of commonly known reference genes in mammalian tissues (Slagboom *et al.*, 1990; Touchberry *et al.*, 2006; Uddin *et al.*, 2011). In

addition, the use of single reference gene may result in erroneous expression (up to 3-fold change) of gene of interest (Vandesompele *et al.*, 2002). Hence, chapter 3 determines, for the first time, a set of reference genes that can be used for relative gene expression studies in granulosa cells of aged cows. For this purpose, granulosa cells were collected from a broad range of physiological conditions (Fig. 3.1). Findings of chapter 3 revealed that mRNA expression of commonly known reference genes (GAPDH and ACTB) did not alter due to maternal or follicular aging (Fig. 3.1). However, based on the comprehensive ranking, UBE2D2 and EIF2B2 were relatively more stable compared with GAPDH and ACTB (Fig. 3.2). In addition, combination of four genes (UBE2D2, EIF2B2, GAPDH and SF3A1) was recommended to normalize the expression of the gene of interest for maternal aging studies (Fig. 3.3) and used for all reported studies (chapter 4, 5 and 6). Hence, this study identified for the first time a combination of the reference genes that are suitable for the study of relative gene expression in granulosa cell during the maternal aging.

Finally, this study demonstrates that maternal age influences the transcriptome of granulosa cells of the dominant follicles and leads to altered response of the granulosa cells during follicular development. Age-associated changes in the transcriptional activity of granulosa cells are more overt in preovulatory follicle 24 h after LH treatment as compared to the dominant follicle at the time of selection.

8 CHAPTER 8: GENERAL CONCLUSIONS

This section summarizes the main findings of the studies in thesis. One of the major strength of the microarray experiments is that they generate a large number of hypotheses for testing and clinical implementation. Therefore, the conclusions of the microarray studies (chapter 4, 5, and 6) should be considered as proposed hypotheses that require further research.

8.1 Selection of stable reference gene for granulosa cells.

a) The mRNA levels of GAPDH or ACTB in granulosa cells of dominant follicles were relatively consistent during maternal aging or follicular aging.
b) Considering the comprehensive ranking analyses, GAPDH and ACTB were relatively less stable than UBE2D2 and EIF2B2.
c) Geometric mean of four genes, UBE2D2, EIF2B2, GAPDH and SF3A1, is recommended for normalization of relative gene expression in granulosa cells of dominant and FSH-stimulated follicles.

We conclude that GAPDH and ACTB mRNA levels do not change due to maternal or follicular aging; however, UBE2D2 and EIF2B2 are relatively more stable.

8.2 Maternal age related changes in transcriptome of granulosa cells of dominant follicle at selection.

A total of 169 genes/isoforms are differentially expressed in granulosa cells of aged cows than young cows. Maternal age in granulosa cells is associated with

a) decreased expression of transcripts related to control of gonadotropins (↓FST, ↓INHBA, ↓INHBB), and the gonadotropin-induced changes in the cytoskeleton

(↑TPM2, ↑ACTG2, ↓Tubulins beta) and extracellular matrix (↓TNFAIP6, ↓VCAN),

b) decreased expression of transcripts related to lipid metabolism (↓LDLR, ↓SCD) and steroidogenesis (↓CYP19A1),cell proliferation (↓PCNA), cell cycle control and checkpoints (↓CHEK1, ↓CENPE) and intercellular communication (↓GJA1).

c) increased expression of transcripts related to oxidative stress response (↑VNN1, ↑VNN2 and ↑GPX3).

We propose that granulosa cells collected at the time of selection of the dominant follicle exhibit age related changes in the transcriptome that result in compromised phenotype of the dominant follicle.

8.3 Maternal age related changes in transcriptome of bovine granulosa cells of preovulatory follilce 24 h after LH treatment.

A total of 1340 genes/isoforms are differentially expressed in granulosa cells of the preovulatory follicle (24 h after LH treatment) from aged vs. young cows. Maternal age in granulosa cells is associated with:

a) slow or suboptimal response to LH (↑RGS2, ↑SERPINE2, ↑PTGS2),

b) delayed synthesis of progesterone (↑STAR, ↑HSD3B2, ↑NR5A2, ↑NR4A1) and low levels of intrafollicle progesterone in aged cows than young and middle-aged cows.

c) delayed terminal differentiation of granulosa cell (↑TNFAIP6, ↑GADD45B, ↓VNN1),

d) prolonged interval to ovulation after prostaglandin treatment in aged cows than young cow.

We propose that changes in transcripts of granulosa cells of preovulatory follicle indicate delayed or suboptimal ovulatory response and decreased progesterone synthesis in aged cows that will result in compromised oocyte competence and corpus luteum development.

8.4 Changes in transcriptome of granulosa cells between the time of dominant follicle selection and 24 h post LH in young vs. aged cattle

Fewer genes/isoforms are differentially expressed in aged cows between the dominant follicles than those from young cows i.e. 1209 vs. 2260. In comparison to young, granulosa cells of preovulatory follicle vs. dominant follicle in aged cows reveal

a) delayed organization of cytoskeleton and cytoplasm (↑ARPC2/5, ↑VCL, ↑VASP, ↑WASF1, and ↑VIPF1),
b) inefficient lipid and cholesterol metabolism (↑SERBF 1/2, ↑LDLR, ↑NPC, ↑INSIG1, ↑ SCD, ↑HMGCR, ↑HMGCS1, ↑ CYP51A1, ↑ACDY, and ↑SQLE),
c) late differentiation and (↓PDGFA, ↓EGR1, ↓NAR5A2) and higher proliferation (↑PCNA),
d) higher oxidative stress and free radical scavenging (↑GSTA1, ↑GSTA4 and ↑SOD2).
e) That the magnitude of change in expression of transcripts is greater in young than aged cows.

We propose that maternal age alters the gene expression of granulosa cells of the dominant follicles in the follicle phase-specific manner and fewer genes are expressed in aged cows than young cows during follicular development due to decreased nucleic acid metabolism.

Overall the studied hypothesis that fewer transcripts in granulosa cells are expressed at the time of dominant follicle selection than preovulatory follicle stage in aged vs. young cows is supported. In addition, maternal age alters the gene expression of the granulosa cells of dominant follicles.

8.5 Future Directions

Results in this study are primarily based on the relative gene expression analysis of granulosa cells, intrafollicular hormone analysis and follicular dynamics between aged and young cows. Future studies may involve protein analysis of the corresponding genes to better understand the age associated changes related to delayed ovulatory response, decreased steroidogeneis and oxidative stress response. Further, gene expression and protein analysis of the theca cells will provide further insight into granulosa-theca interactions and characterize their relative impact of maternal age on follicular environment. In addition, spatio-temporal pattern of the genes and protiens before and after LH treatment of granulosa cells of the dominant follicles from aged cows is also warranted to better understand the age associated delay in ovulation. Another aspect of future research may be the evaluation of the oocyte collection timings for in vitro fertilization in aged cows.

Based on the relative gene expression analysis in aged cows, strategies may be devised to improve the response of follicular cells to the gonadotropin (FSH or LH) treatment. Therefore, further research related to lipid/cholesterol processing and the production of androgens by follicular cells of aged cows may be useful. In addition, research related to the epigenetic changes in follicular cells may help to devise strategies for improving outcomes of fertility treatments in aging patients.

9 CHAPTER 9: BIBLOGRAPHY

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10 APPENDEX A: SUPPLEMENTARY TABLES

Supplementary Table 10.1 List of differentially expressed gene in granulosa cells of aged vs. young cows at the time of dominant follicle selection. Genes have been clusterd according to their molecular function.

Function	Gene Symbol	Description	Fold change
Cell proliferation, Cell cycle, DNA replication and spindle check points	CHEK1	checkpoint kinase 1	-2.1
	TFDP1	transcription factor Dp-1	-2.0
	SMARCE1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin e1	2.1
	IRF7	interferon regulatory factor 7	2.2
	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	-2.4
	MAD2L1	MAD2 mitotic arrest deficient-like 1	-2.1
	MAD2L2	MAD2 mitotic arrest deficient-like 2	2.2
	PBK	PDZ binding kinase	-2.4
	PSMB10	proteasome (prosome, macropain) subunit, beta type, 10	2.1
	ARAF	A-Raf proto-oncogene serine/threonine-protein kinase	2.0
	PCNA	proliferating cell nuclear antigen	-2.5
	MYH11	myosin, heavy chain 11, smooth muscle	2.4
	CENPE	centromere protein E, 312kDa	-2.3
	MCM4	minichromosome maintenance complex component 4	-2.4
	PTTG1	pituitary tumor-transforming 1	-2.0
	NUSAP1	nucleolar and spindle associated protein 1	-2.1
	HAT1	histone acetyltransferase 1	-2.1
	H3F3B	H3 histone, family 3B	-2.1
Lipid/cholesterol/Steroid Synthesis	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	-2.3
	CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1	-4.5
	SCD	stearoyl-CoA desaturase	-3.2
	HMGCSI	3-hydroxy-3-methylglutaryl-CoA synthase 1	-2.2
	SC4MOL	sterol-C4-methyl oxidase-like	-2.4
	CD36	Fatty acid translocase	2.4
	LDHA	actate dehydrogenase A	-2.9
	INSIG1	insulin induced gene 1	
	LRP8	low density lipoprotein receptor-related protein 8	-3.2
	STRA6	stimulated by retinoic acid gene 6 homolog	-2.1

Gonadotropin regulation	INHBA	inhibin, beta A	-2.4
	INHBB	inhibin, beta B	-2.5
	FST	Follistatin	-2.5
Oxidative stress	GPX3	Glutathione peroxidise 3	2.3
	VNN1	Vanin 1	5.5
	VNN2	Vanin 2	3.8
Citric acid cycle and mitochondrial electron transport	IDH3A	isocitrate dehydrogenase 3 (NAD+) alpha3	-4.3
	LDHA	actate dehydrogenase A	-2.9
Amino acid synthesis	OAT	ornithine aminotransferase	-2.7

Supplementary Table 10.2 List of top ten up- and down-regulated genes in granulosa cells of preovulatory follicle (24 h after LH treatment) of aged vs. young cows (≥2-fold change in expression; $P \leq 0.05$, as assessed by microarrays). Function(s) of each gene in ovarian follicle is provided from literature.

Expression	Gene Symbol	Fold Change	Function in follicle
Up regulated	TNFAIP6	4.8	Granulosa cell Differentiation, luteinization, inflammation, extracellular matrix remodelling (Sayasith *et al.*, 2008)
	GEM	4.5	Signal transduction in primary human granulosa cells (Sasson *et al.*, 2004)
	CRISPLD2	4.3	Unknown
	GFRA1	3.5	Folliculogeneis, Primordial follicle activation (Farhi *et al.*, 2010) predicted role in oocye maturation, and ovulation induction (Dole *et al.*, 2008)
	NUDT11	3.5	Unknown; upregulated after LH treatment of bovine granulosa and thecal cells (Christenson *et al.*)
	MRO	3.4	Unknown; upregulated after FSH treatement of mouse granulosa cells (Deroo *et al.*, 2009)
	ASB9	3.4	Unknown
	CLDN11	3.2	Unknown
	SLC39A8	3.0	Unknown; upregualted 6 h post LH treatment of bovine granulosa cells (Gilbert *et al.*, 2011)
	GFPT2	3.0	Upregualted as indication of oxidative stress in granulosa cells (Nivet *et al.*)
Down regulated	VNN1	-4.4	Oxidative stress in granulosa cells (Nivet *et al.*; Khan *et al.*, 2012)
	PERP	-4.4	Apoptosis (Nivet *et al.*)
	PPL	-4.4	Unknown
	EPCAM	-4.2	Unknown; related to ovarian surface epithelium (Gava *et al.*, 2008),
	KRT19	-4.0	Unknown; In inhibin deficient mice KRT19 down regulated in granulosa cells representing defective gene expression (Nagaraja *et al.*, 2010).
	FXYD3	-4.0	Unknown; modulates ion channels of oocyte
	TMEM79	-3.9	Unknown
	WWC1	-3.8	Unknown
	GIPC2	-3.8	Unknown
	DSG2	-3.6	Unknown; Related to adhesion and junctions (Mora *et al.*, 2012)